



PHD

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**THE DEVELOPMENT OF INDUSTRIALLY-RELEVANT LIPIDS FROM
RHODOTORULA SPECIES AS A FEEDSTOCK FOR FUELS AND COMMODITY
PRODUCTS.**

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Chemical Engineering

October 2014

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Signed on behalf of the Faculty of Engineering

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Abstract

Lipids are becoming an increasingly important chemical feedstock for the manufacture of biofuels, bioplastics, care-products and as a food source. Many of these consumer products are derived from petroleum resources, and therefore finding suitable replacements is a key engineering challenge. While *first generation* lipid feedstocks have shown potential to displace some fossil fuel use, lipids produced from current sources such as oil crops, cannot realistically meet the demand for these uses sustainably. One alternative is to produce microbial oils from oleaginous yeast. These have many advantages such as high growth rates, year-round productivity and high lipid yield.

The fatty acid profile of the lipids is extremely important in determining their eventual use. Oils high in oleic acid such as rapeseed oil are the most suitable biodiesel feedstock and also offer the highest potential for further chemical upgrading to polymers, higher value chemicals or aviation fuels. Alternatively, to replace palm oils in the cosmetic or food industries, high levels of saturated lipids are necessary. *Rhodotorula sp.*, can produce high yields of lipid and has a simple fatty acid profile, composed mainly of C₁₆ and C₁₈ fatty acids. Using Design of Experiments it was shown that the fatty acid profile of *R. glutinis* could be tailored towards a desired application. A high culture temperature and high nitrogen ratio yielded mainly unsaturated oil, whereas a low culture temperature and high glucose loadings gave a more saturated profile. On transesterification, the oil high in monounsaturated esters yielded biodiesel with fuel properties akin to rapeseed methyl ester (RME), whereas the oil high in saturates was found to be suitable as a substitute for palm oil. In contrast, the lipid profile for *R. minuta* showed no such fluctuation.

One of the drawbacks to the commercialisation of this technology is the high production costs involved. Low energy ultrasound is known to have a positive effect on both biomass and ethanol production in *S. cerevisiae*. In an attempt to reduce processing costs, intermittent ultrasound with *R. glutinis* was undertaken to aim to improve glucose conversion efficiencies. Sonication was found to have no positive

effect on the biomass or lipid accumulation when applied in the exponential phase of *R. glutinis* growth. However, on applying the sonication in the stationary phase, a beneficial impact was observed with the lipid coefficient being increased by 24%.

While it is unlikely to be economic to produce lipids from refined sugars, inexpensive carbon sources such as lignocellulosic hydrolysates or waste streams offer a promising alternative. Microbial growth on these feedstocks can however be challenging, due to the large range of sugars present in the hydrolysates as well as toxic compounds formed during the sugar extraction process. The potential of two biomass hydrolysates: depolymerised *Miscanthus* and household food waste were investigated, alongside the effects of the model inhibitory compounds and sugar substrates on the growth of *Rhodotorula sp.* While the *Miscanthus* hydrolysate was deemed unsuitable as a feedstock for lipid production, acid-hydrolysed food waste produced a promising feedstock for *Rhodotorula sp.* Biomass yields of approximately 10 g/L were produced for *R. minuta* and *R. glutinis*, with the resulting lipid profile being approximately 65% oleic acid (18:1) for both species.

One obstacle for lipid production from oleaginous microbes are the energy costs associated with the extraction and subsequent conversion into biodiesel (FAME). A one-step method to produce FAME by combining lipid extraction from *R. glutinis* using a microwave reactor with acid-catalysed transesterification was developed. Over 99% of the lipid was extracted using 25 wt.% H₂SO₄ over 20 min at 120 °C. At higher catalyst loadings, similar yields were achieved at a reaction time of 30 s. Equivalent yields of FAME were achieved compared to the traditional method of Soxhlet extraction, run with the same solvent system for 4 h. Under the best conditions, the energy required by the microwave was less than 20% of the energy content of the biodiesel produced. Finally, the energetics of the conversion of household food waste to oil (SCO process) using *Rhodotorula sp.* was compared to that of the anaerobic digestion (AD) of food waste. Oil production alone was deemed energetically unfeasible. However, a coupled SCO and AD plant may have economic viability as a waste-to-energy route, especially for the production of bulk commodities such as jet fuel, in which the energy generated from the methane can be used to power the SCO process.

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Abbreviations and Acronyms

AMP	Adenosine monophosphate
ASTM	American society for testing and materials
ATP	Adenosine triphosphate
ATP-CL	ATP-citrate lyase
C/N	Carbon-to-nitrogen ratio
c.f.u	Colony forming units
CHCl ₃ / CDCl ₃	Chloroform / deuterated chloroform
CHP	Combined heat and power
CO	Carbon monoxide
CO ₂	Carbon dioxide
Co-A	Co-enzyme A
CSTR	Continuous stirred tank reactor
DO	Dissolved oxygen
d.wt	Dry weight
E.U.	European Union
EMP	Embden Meyerhof-Parnas pathway
EU ETS	European Union emissions trading scheme
FAAE	Fatty acid alkyl ester
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FFA	Free fatty acid
g/L	Grams per litre
GC-MS	Gas chromatography mass spectrometry
GHG	Greenhouse gas
GM	Genetically modified / genetic modification
HEFA	Hydroprocessed esters and fatty acids
HMF	5-hydroxymethylfurfural
LHV	Lower heating value
MeOH	Methanol
Mol.%	Mole percent
Mtoe	Million tonnes of oil equivalent
NCYC	National collection of yeast cultures (Norwich, UK)
NO _x	Nitrous oxides
OD	Optical Density
ppm	Parts per million
PPP	Pentose phosphate pathway
PUFA	Polyunsaturated fatty acid
SCO	Single cell oil
SD	Standard deviation from the mean
Sp.	Species
TCA	Tricarboxylic acid
TG	Triacylglyceride
v/v	Volume/volume
w/v	Weight / volume
wt.%	Weight percent

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CHAPTER 1:

INTRODUCTION

“You have to know the past to understand the present.”

Dr. Carl Sagan

1.1 Opening remarks

The need to move away from our dependence on fossil fuels has been well documented. The finite nature, geopolitical uncertainty and negative environmental impacts of fossil fuels, make the replacement of petrochemical oil sources with sustainable alternatives one of the biggest challenges in the 21st century. One alternative is to produce oil from microbial oils such as yeast. This chapter delivers an overview of the current literature, providing background information on the production of single cell oils as well as their application within industry as a feedstock for fuels and commodity products. The literature on lipid production from *Rhodotorula* sp. is also explored. The chapter concludes with the aims and objectives surrounding the work conducted. The transport fuel application of this literature has also contributed towards a review article published in 2014 which can be found in Appendix C (p 203):

C. J. Chuck, F. Santomauro, L. A. Sargeant, F. Whiffin, T. Chantasuban, N.R. A. Ghaffer, J. L. Wagner, R. J. Scott. **2014**, Liquid transport fuels from microbial yeasts – current and future perspectives. *Biofuels*. 5 **(3)** 293-311.

1.2 History

Since the first commercially-successful oil well was drilled in 1859, we have been aware of its finite quantities. While its discovery was initially viewed as a replacement for heating and lamp oil, petroleum became the dominant road transport fuel within 70 years of it first being extracted. In 1890, encouraged by the low efficiencies surrounding steam engines and the low value of *waste* middle-distillates (e.g. kerosene and diesel), Rudolf Diesel (1858-1913) developed the diesel engine, which along with the fuel that powers it, now bears his name. Shortly after its development, at the 1900 World's Fair in Paris, the use of peanut oil was demonstrated in a diesel engine. However, this was at the request of the French Government due to the plentiful supply of peanut oil in African colonies to enable the colonies to be self-sufficient;⁴ Diesel himself designed the engine to be fuelled by mineral oil. In later years he did however become an advocate of vegetable oils for use in vehicle engines.⁴

During World War II, vegetable oils received considerable interest due to their potential as an emergency fuel supply as uncertainty surrounding petroleum oil increased. At the time, ships were also switching from coal-fired vessels to oil-fired ones, increasing the demand for oil. This meant that many nations prevented the export of vegetable oils to ensure supplies in the event of a fuel shortage, as well as the cracking of fuel to produce gasoline and kerosene fractions. A considerable amount of research into alternative oil sources and blending with petroleum fuels was also undertaken at this time. However, once oil prices stabilised after the war, oil quickly became a cheap and abundant supply of energy, and interest into renewable alternatives declined. Interest was however reignited during the energy crisis of the 1970s and early 1980s, when there was again uncertainty surrounding the supply of mineral oil. Still today, geopolitical instability is one of the major motivators for research into alternative energy sources. For example, it is still being debated as to whether the true motive of the Iraq war (2003-2011) was to secure global oil supplies.⁵

1.3 Current outlook

The use and production of crude oil is one of the major drivers of the modern global economy. It is believed that proven oil, gas, coal and uranium reserves will be exhausted in 60-80 years.⁶ Even assuming there is a stable global population of 9 billion people after 2050, the *ultimately recoverable* reserves are only estimated to last until 2084 if the rate of economic growth is 3% per annum.⁷

In a typical barrel of oil, approximately 80% is upgraded into light and middle distillates such as gasoline, diesel and jet fuel; with around 12% of heavy distillates being used in bitumen, lubricating oils and waxes. The remainder is used in many different chemical processes, but it is vastly dominated by the plastics industry. Each one of these applications requires different physical properties of the oil. Within a traditional oil refinery, the process involves cracking, separation and further hydrotreatment of crude oil. This yields fractions of differing chain lengths, saturation and branching, and thus varying physical properties. However, the rising cost and finite quantities of current oil resources, coupled with the environmental

concerns surrounding the use of petroleum have prompted the need for sustainable alternatives.

1.4 The transition to a bio-based economy

In order to establish solutions to reduce the rapid consumption of fossil resources, the transition to a sustainable bio-based economy encompassing bio-based products, bio-energy and bio-fuels is considered to be essential.⁸ In the European Union, the *Biofuels Directive* (2003/30/EC) adopted in 2003 targeted 5.75% of diesel transport to be biomass-derived by December 2010.⁹ This was adapted in the 2009 *Renewable Energy Directive* (2009/28/EC) in which the target is 10% use of renewable energy in the EU transport sector by 2020.¹⁰ The US Department of Energy has also set a target of 25% of industrial organic chemical feedstocks to be derived from bioresources by 2025.⁹ Similarly, €3.7 billion is to be invested in the European economy between 2014 and 2024 to develop the bioeconomy sector.¹¹ In order to meet these targets, it has been suggested that businesses need to be based around a biorefinery model, in which renewable plant material is converted into higher-value fuels, chemicals and materials (Figure 1.1).¹² As such, considerable attention has been attached to raw materials that exploit the synthetic capabilities of nature.¹³

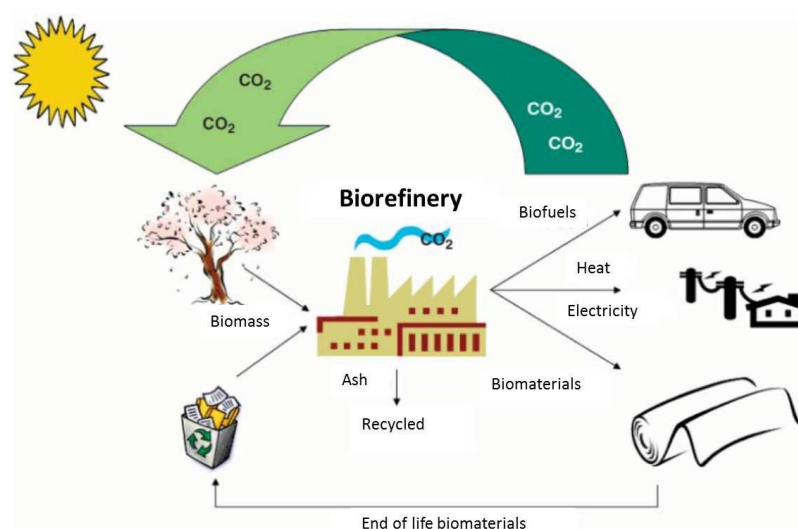


Figure 1.1: A fully integrated biorefinery model providing bio-fuels, bio-materials and bio-energy for sustainable technologies (taken from Ragauskas *et al.*).⁹

Vegetable oils and animal fats currently make up the greatest proportion of renewable raw materials in the chemical industry. Globally, around 160 billion tonnes of plant oils are produced per year derived from plant seeds such as soybean, rapeseed and palm oil.¹⁴ The increasing demand in vegetable oils to meet renewable targets is causing increasing pressure on the availability of these oils worldwide. The oils can be readily converted to suit a broad range of chemical applications, thus increasing the demand.¹³ This includes fuel molecules for the road transport and aviation industries, as well use in polymer applications, lubricants, and home & personal care products. The choice of the oil feedstock is paramount to the economic viability of the end product, as it can account for over 75% of the overall cost of the process.¹⁵

1.4.1 Lipid structure and nomenclature

Lipids are broadly defined as fats, oils or waxes that are insoluble in water but are soluble in many organic solvents. Lipids that are solid or semi-solid at room temperature are generally classified as fats, and occur predominantly in animals. In contrast, lipids that are liquid at room temperature are generally classified as oils, originating largely from plant seeds. Lipids are predominantly composed of triacylglycerides (TGs), which serve as the main storage of energy and carbon in plant and animal cells. TGs consist of three fatty acid molecules (alkyl chains) esterified to a glycerol backbone (Figure 1.2).

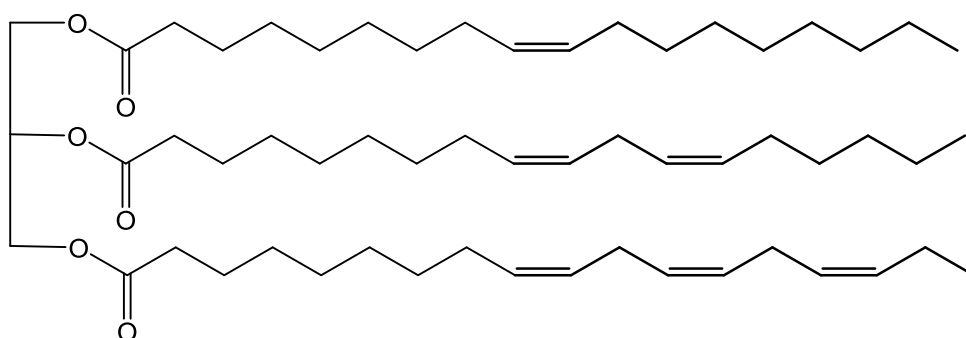


Figure 1.2: Representation of triacylglyceride found in plant oils, comprised of oleic acid (top), linoleic acid (middle) and linolenic acid (bottom).

The chain length and degree of saturation of the fatty acid chains vary considerably, and depends on the source of the feedstock as well as the growth conditions of the

animal or plant from which the lipid is obtained. In terrestrial plants, the length of the alkyl chain on the triacylglycerides is generally between 12-20 carbons, with up to 3 double bonds. It is this high level of polyunsaturation that results in the lipid being an oil rather than a fat. In microbial oils there is more variation with fatty acid chains up to 26 carbons and up to 6 double bonds being present, especially in microalgal oils (Table 1.1).

The fatty acid chains are referred to with a first number depicting the number of carbons in the aliphatic chain and a second number, following a colon, denoting the number of double bonds. The double bonds are found almost exclusively in the *cis* orientation and are spaced three atoms apart (e.g. 6,9,12-linolenic acid). The most common lipids in nature are methyl palmitate (16:0), methyl palmitoleate (16:1), methyl stearate (18:0), methyl oleate (18:1), methyl linoleate (18:2) and methyl linolenate (18:3).

The length and degree of unsaturation of the alkyl chain greatly influence the physical properties of the resulting oil. Generally, the viscosity of the oil increases with an increasing chain length. As the degree of saturation within the oil increases, so too does the viscosity. This higher degree of saturation also confers a greater oxidative stability. Conversely, as the amount of polyunsaturation increases, the oxidative stability of the oil decreases but the viscosity is much lower. This relationship between the length and degree of saturation on the physical properties of the oil, necessitates that the oil should be carefully selected for its desired application.

Table 1.1: Typical fatty acid methyl ester (FAME) profiles for common oleaginous organisms.

Organism	Lipid Content (% w/w)	FAME (% of total lipid) [†]										Other
		C14:0	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3	C20:5	
Microalgae¹⁶												
<i>Chlamydomonas reinhardtii</i>		2	32	2	2	2	-	18	11	22	-	C12:0 = 2, C15:0 = 2, C18:4 = 0.7. Trace amounts of: C14:1, C20:2 C15:0 = 2, C17:0 = 1, C20:4 = 4.5. Trace amounts of: C14:1, C20:2, C20:3.
<i>Chaetoceros calcitrans</i>	3-17	19	26	28	-	-	3	5	Trace	-	7	
<i>Dunaliella salina</i>	16-44	1	19	2	2	7	2	5	6	39	Trace	
<i>Nannochloropsis oculata</i>	2-10	4	25	23	1	1	2	6	4	4	28	
Yeast¹⁷												
<i>Lipomyces starkeyi</i>	61-68	-	467	3	-	-	10	38	1	Trace	-	
<i>Rhodospiridium toruloides</i>	55-67	-	31	1	-	-	12	48	4.8	Trace	-	
<i>Yarrowia lipolytica</i>	22-43	-	13	10	-	-	9	53	13	1	-	
<i>Rhodotorula glutinis</i>	22-72		37	-	-	-	3	47	8	Trace	-	
<i>Cryptococcus curvatus</i>	25-50	-	24	Trace	-	-	16	46	9	2	-	
Plants												
Rapeseed (<i>Brassica napus</i>) ¹⁸	~45	-	2	-	-	-	2	65	21	10	-	
Soybean (<i>Glycine max</i>) ¹⁸	~20	-	10	-	-	-	4	30	48	8	-	
Palm (<i>Elaeis guineensis</i>)	~50	-	40	-	-	-	5	45	10	-	-	
Linseed (<i>Linum usitatissimum</i>) ¹⁹	~42	-	5	-	-	-	4	22	17	52	-	
Sunflower (<i>Helianthus annuus</i>) ¹⁹	~50	-	17	-	-	-	4	26	53	Trace	-	
Cocoa (<i>Theobroma cacao</i>)		4	26	1	-	-	30	35	4	-	-	

[†] Values are indicative of a mean value, but can vary significantly depending on strain and growth conditions.

1.4.2 Microbial lipids

The current method of lipid production using feedstocks such as oil crops, waste cooking oil and animal fats is insufficient to satisfy the future demand for the fuel and chemical industries. *First-generation* feedstocks from edible oils such as rapeseed, soybean and palm oil have attracted considerable public criticism due to concerns surrounding land use and food security. The development of *second-generation* feedstocks from non-edible resources, such as jatropha, offered potential as a sustainable alternative, but the technology surrounding the conversion of such feedstocks has not reached viability for commercial exploitation. To be a suitable component of a future biorefinery, the lipid feedstock must be economically viable and require minimal agricultural land use. The lipids should also demonstrate superior environmental benefits compared to crude oil whilst maintaining suitable properties for the application. Foremost, to make a significant contribution to addressing the demand, the lipids must be capable of being produced in substantial quantities.²⁰ To meet these requirements, *third-generation* feedstocks using microorganisms that can produce fats and oils (oleaginous microorganisms) have been considered as potential alternatives. Oleaginous microorganisms can produce more than 20% (w/w) of microbial lipid in their dry cell mass and is also referred to as single cell oils.¹⁷ The term single cell oils was first used in the 1970s, but has attracted a huge amount of research interest in recent years due to the need for a sustainable oil supply (Figure 1.3). Research into microbial lipids can be traced back as far as 1878, when the fatty acid composition of *Saccharomyces cerevisiae* was reported.²¹ Interest into microbial oils were particularly heightened around the early 20th century, with concerns surrounding the disruption of vulnerable oil supplies due to the commencement of World War I and II, so that alternative oil sources could be produced if necessary. However, problems surrounding scale-up, recovery and extraction were not overcome, and the resulting high-energy biomass was fed only to the army horses.³

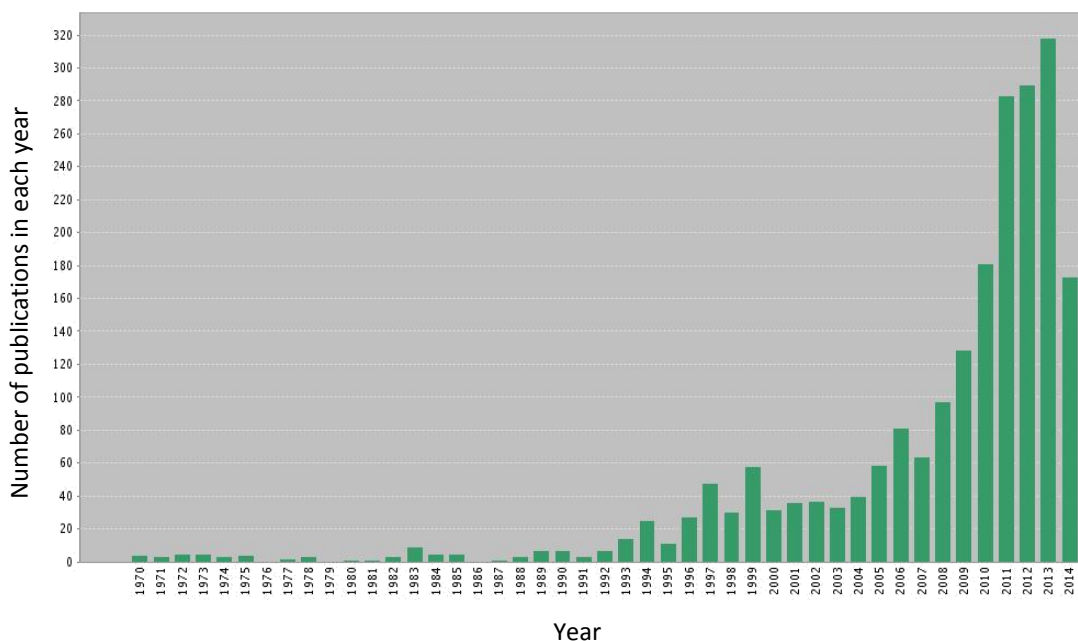


Figure 1.1: Number of publications including *single cell oil* in the title from 1970-2014. Source: Web of Knowledge, 17 June 2014. The exponential increase in the number of publications from the late 1980's reflects the increasing interest into alternative oil sources, underpinned by the need for a sustainable oil supply.

A wide array of microorganisms can produce lipids, including microalgae, bacteria, fungi and yeast. However, to date only microalgae and a small number of oleaginous yeast have been investigated. Similarly to plant oils, microbial oils are mainly composed of triacylglycerides, stored in discrete lipid bodies,²² but can also consist of free fatty acids, other neutral lipids (such as mono- and di-acylglycerides and steryl-esters), sterols, polar lipids (e.g. phospholipids, sphingolipids, glycolipids), and hydrocarbons.¹⁷ The mono- and di-acylglycerides, along with the polar lipids are generally found in the plasma membrane.²¹ Compared to other vegetable oils the production of microbial oil has many benefits. The biomass doubling time within exponential growth can be as short as 40 min and the oil content within the cell can exceed 80% by weight of dry biomass, meaning that oil productivities can be considerably higher compared to oil crops. The cultivation of oleaginous microorganisms is also less labour intensive, less affected by location, season and climate, and are easier to scale up than terrestrial crops.²³ For example, it has been estimated that the land area needed to produce the same volume of transportation fuel is 180 times greater for soybean and 40 times greater for

jatropha than for microalgae; assuming the microalgae is grown in a photobioreactor to a concentration of 4 kg/m^3 and an oil content of 50% (d.wt).²⁴

1.5 Oleaginous microalgae

The majority of work into microbial lipid production has focused on autotrophic microalgae. Microalgae are unicellular, photosynthetic organisms that convert CO_2 into biomass and lipids. Microalgal lipids have mainly been researched for the production of biodiesel, with many reports and articles assessing the viability of biodiesel production in comparison with other available feedstocks.²⁴⁻²⁸ Microalgae grow in an aquatic environment, and have been shown to grow in salt water and wastewater.²⁵ Microalgae also sequester CO_2 , biomass is produced in the process of CO_2 fixation through photosynthesis. Whilst this also occurs in terrestrial crops, microalgae have been shown to exhibit 10-50 fold greater efficiency to fix CO_2 than oil crops.²⁹ It is due to the ability of microalgae to be able to fix CO_2 using the energy from the sun that has resulted in a huge amount of interest in microalgae, with the belief that oil could be produced cheaply from free resources. However, while the production of algal oils and the subsequent conversion into useable fuels has been demonstrated repeatedly,^{24,30-31} the commercialisation of this technology still remains elusive.

1.5.1 Heterotrophic algal growth

By comparing fifteen published articles based on the life cycle analysis of oil production from microalgae, Sills *et al.* concluded that only high microalgal productivity ($34\text{-}50 \text{ g}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$) combined with extracting the oil from wet biomass would yield an economically favourable process.³² In order to achieve these yields, heterotrophic growth using organic carbon as an energy source has received considerable attention for microalgal growth. This utilises fermentation technology in the form of stirred tank reactors. Mixotrophic growth, using CO_2 and organic carbon in the presence of light is also possible.

Different species of microalgae have been reported to be able to grow heterotrophically, including *Chlorella sp.*, *Haematococcus sp.* and *Chlamydomonas reinhardtii*. Biomass yields from heterotrophic growth are significantly higher than

those achieved for autotrophic growth. For example, heterotrophic growth of the microalga *Chlorella zofingiensis* yielded a cell density of 10.1 g/L compared to 1.9 g/L when grown under autotrophic growth conditions.³³ For this reason, heterotrophic growth has been employed as an industrially viable method for lower value applications. By growing microalgae heterotrophically, the U.S. biotechnology company, Solazyme, are producing drop-in fuel replacements for the road transport (Soladiesel_{RD}[®]), marine (Soladiesel_{HRF-76}[®]) and aviation (Solajet[®]) sectors from microalgae.³⁴

While considerably better than photoautotrophic growth, the heterotrophic growth of microalgae still has several limitations. Firstly, most microalgae are photosynthetic organisms, and thus the number of heterotrophic species available without genetic modification is limited. Microalgae also have a broad lipid profile (Table 1.1), which while desirable in niche applications, could hinder economic development due to variations in the feedstock. Finally, the economics surrounding the cost of the production of algal oil is unfeasibly high. This has been estimated to be around US \$7000/tonne compared to US \$1500/tonne for rapeseed oil.³⁵

1.6 Yeast in industrial biotechnology

In terms of economics and cultivation methods, the production of yeast is similar to growing microalgae heterotrophically.³⁵ However, yeast exhibit several advantages over microalgal feedstocks for lipid production. Yeast are heterotrophic organisms, and as such, all of their energy for cellular respiration and division is acquired from an organic carbon source. Yeast do not require light, have shorter doubling times, and they can reach much higher cell densities (10-100 g/L in 3-7 days) when compared to microalgae.³⁶ Yeast are also less susceptible to viral infection than microalgae and can be grown in low pH conditions which can reduce the likelihood of bacterial contamination.²¹ Yeast are particularly versatile as hosts for genetic modification, which has resulted in yeast being the principal model eukaryotic organism utilised for fundamental research.³⁷ In addition to the oils, yeast also produce a range of co-products. These co-products are especially important when investigating the production of relatively low value commodity products such as

fuels, as these co-products are economically attractive to a potential biorefinery plant. Co-products include biologically-derived surfactants (e.g. sophorolipids from *Rhodotorula bogoreinsis*)³⁸⁻³⁹ which are biodegradable, as well as high value carotenoids such as astaxanthin, from *Phaffia rhodozyma*.⁴⁰ Solazyme, the Californian-based microbial oil producer, have also applied for several patents surrounding yeast oil-to-fuel; evidence of strong industrial interest in oleaginous yeast.²¹

Yeast have been exploited for the production of beer, wine and bread for many centuries, but it is only in the last few decades that the extent of the biotechnological use of yeast has been understood. The biotechnological potential of yeast has been exploited for the production of food, medical, scientific and agricultural purposes (Figure 1.4).⁴¹ The productivity of the economic revenue obtained from yeast products exceeds that of any other group of industrial microorganism.^{37 51} Although there are over 1600 known yeast species, the vast majority of research, both from an applied and fundamental perspective has been undertaken with yeast from the *Saccharomyces* genus, especially the ethanol-producer, *S. cerevisiae*. This is partly due to its resilience to industrial conditions,⁴² and was named the *first domesticated organism* for its biotechnological applicability. The majority of the research into the genetic modification of *S. cerevisiae* has been to improve xylose assimilation (due to the prevalence of xylose in depolymerised lignocellulose feedstocks), enhancing the ethanol yields or to produce alternative fuel molecules such as butanol, farnesane or isoprene.⁴²

Perhaps the most well-known of the non-*Saccharomyces* yeast are of the ascomycetes phylum, which are particularly important for food (single cell protein) and bioethanol production.⁴³ Some ascomycete yeast are also known to be oleaginous. These include *Candida*, *Cyberlindnera*, *Geotrichum*, *Kodamaea*, *Lipomyces*, *Magnusiomyces*, *Metschnikowia*, *Trigonopsis*, *Wickerhamomyces*, and *Yarrowia* genera.⁴⁴⁻⁴⁷ In contrast, basidiomycetes yeast have only been studied extensively in the last few decades, with very little industrial exploitation. A number of these yeasts do however have significant industrial potential especially regarding enzyme manufacture, bioremediation and the production of primary and

secondary metabolites, such as lipids (Figure 1.4).⁴¹ Approximately 60% of oleaginous yeast species are found within the basidiomycete genera,²¹ and include: *Cryptococcus*, *Guehomyces*, *Leucosporidiella*, *Pseudozyma*, *Rhodospiridium*, *Rhodotorula* and *Trichosporon*.^{44,48-52} Though species-specific, most oleaginous yeast can produce lipids from a variety of carbon sources including glucose, xylose and glycerol. Work is also underway to culture some oleaginous yeast on waste streams such as depolymerised cellulose. The ability to grow in vitamin-free media is also a common trait of basidiomycete yeast, a characteristic which is of great benefit when looking at reducing production costs.²¹

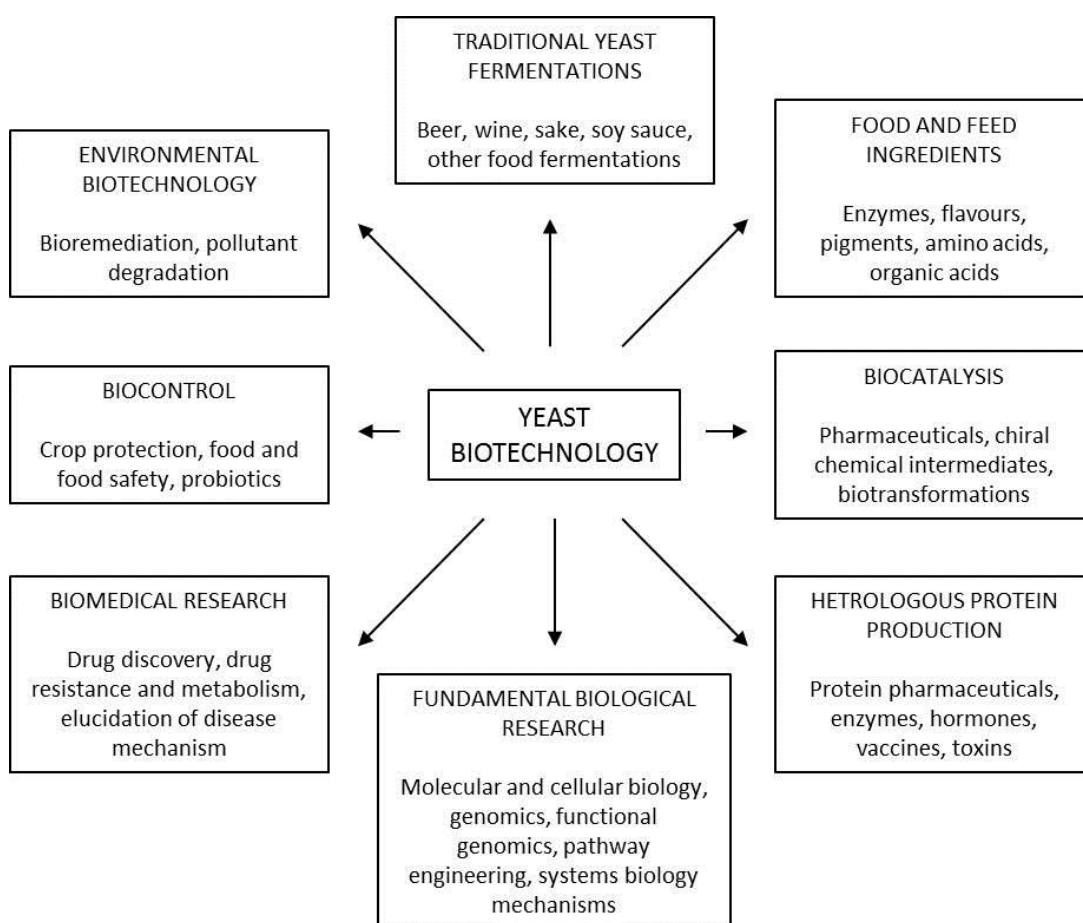


Figure 1.4: Applications of yeast biotechnology (copied from Johnson *et al.*).⁴¹

1.6.1 Oleaginous yeast

Of over 1600 known yeast species, 70 are known to be oleaginous.²¹ Oleaginous yeasts are capable of accumulating intracellular lipids up to 80% of their dry biomass weight in discrete lipid bodies (Figure 1.5).

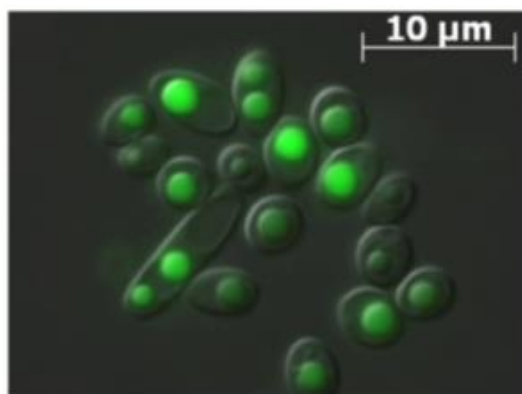


Figure 1.5: Visualisation of the lipid bodies in the yeast *Yarrowia lipolytica* when grown in glucose medium (copied from Thevenieau and Nicaud).¹

In general, yeast lipid is composed of C₁₆ and C₁₈ fatty acids. Oleic acid (18:1) is the principal lipid accumulated in yeast cells, sometimes to higher than 70% (w/w). Palmitic acid (16:0) and linoleic acid (18:2) each constitute around 15-25% (w/w) of the total lipid, whereas stearic acid (18:0) and palmitoleic acid (16:1) are minor components of the oil, found in concentrations of 5-8% (w/w) and less than 5% (w/w), respectively.¹⁷ Polyunsaturated lipids such as α -linolenic acid (18:3) are not commonly synthesised in yeast oils.¹⁷ The relative composition of these lipids can however vary between yeast species (Table 1.2), as well as between different strains of the same species. On the whole, yeast oils have a similar profile to plant-derived oils, such as rapeseed and sunflower oil, making them the ideal replacement for a sustainable feedstock.

Table 1.2: Lipid contents and fatty acid profile of the highest oleaginous yeast.³⁵

Yeast species	Maximum lipid content (% w/w) [†]	Major fatty acids (% w/w)			
		16:0	18:0	18:1	18:2
<i>Cryptococcus curvatus</i>	60	32	15	44	8
<i>Lipoyces starkeyi</i>	65	34	5	51	3
<i>Rhodospiridium toruloides</i>	66	18	3	66	-
<i>Rhodotorula glutinis</i>	72	37	3	47	8
<i>Waltomyces lipofer</i>	64	37	7	48	3

[†]>90% of the lipid is triacylglyceride

1.6.2 Basidiomycota phyla

Basidiomycete yeast are most commonly found in relatively dry, nutrient-poor habitats such as on the surface of leaves and fruits (phylloplane), where the ability to survive periods of famine is essential. Because of this habitat, many basidiomycete yeast are able to survive on a wide variety of sugar sources, sugar alcohols and to a certain extent, lignin components. Generally, basidiomycete yeast can assimilate a broader array of carbon sources than ascomycete yeast species.⁵³ By evolving to store the assimilated sugars as lipid, the yeast are able to ensure survival during periods of deprivation.²¹ Due to their habitat, basidiomycetes yeast also produce a range of interesting products, many of which protect against irradiation and reactive oxygen species, such as carotenoids. Of the basidiomycetes yeast genera, *Cryptococcus sp.*, *Rhodotorula sp.*, *Rhodospiridium sp.*, *Trichosporon sp.*, *Xanthophyllomyces dendrorhous* and *Phaffia rhodozyme* have been identified as species that are of biotechnological importance.⁴¹

1.6.2.1 *Rhodotorula* species

Rhodotorula sp. have been isolated from a range of habitats including plants, soil⁵⁴ and pine glacial environments.⁴⁸ The ability of *Rhodotorula sp.* to be able to grow on biomass is indicative that many *Rhodotorula* species are involved in the natural biodegradation of biomass. For example, Hainal *et al.* demonstrated that *Rhodotorula* species are able to metabolise lignin.⁵⁵ Using lignin separated from wheat straw and Saekanda grass at concentrations of 0.1% and 0.5% (w/v), the authors presented structural changes associated with lignin metabolism and achieved yeast biomass yields of up to 17 g/L (wet weight). The specific *Rhodotorula* species used within this study are however unknown.⁵⁵ Similarly, *Rhodotorula sp.* are known to be able to assimilate compounds present in the breakdown of renewable resources such as furfural, acetic acid and 5-hydroxymethylfurfural (5-HMF).⁵⁶⁻⁵⁷ This property offers exciting industrial potential for the remediation of biomass waste streams.

Rhodotorula sp. have also been researched as bio-control agents. *R. glutinis* was shown to be able to metabolise patulin, a mycotoxin commonly found in apple products,⁵⁸ as well as preventing the formation of blue-grey mould (caused by

Botrytis cinerea and *Penicillium expansum*) on apples.⁵⁹ *R. minuta* was also shown to be an effective biocontrol agent for mango anthracnose (fungal disease of plants).⁶⁰ *Rhodotorula sp.* may also have industrial potential as bioremediation agents. *R. glutinis* has been shown to be effective at absorbing uranium from wastewater streams,⁶¹⁻⁶³ whereas *R. rubra* and *R. creatinivora* are able to metabolise phenol.⁶⁴⁻⁶⁵ Furthermore, enzyme production from *Rhodotorula sp.* has also been investigated. Possibly of most interest is the production of phenylalanine ammonia lyase,⁶⁶⁻⁶⁷ used for the production of L-phenylalanine from *R. glutinis*.⁶⁸

Many *Rhodotorula sp.* are known to produce carotenoids including β -carotene, torulene and torularhodin.⁶⁹ Carotenoids are lipid-soluble pigments, the majority of which are C₄₀ terpenoids.⁷⁰ Carotenoids are of commercial interest due to their use as food colourants and nutritional supplements, but also as natural antioxidants. While other yeast species (such as *Xanthophyllomyces*) are able to convert β -carotene into the high-value antioxidant, astaxanthin, generally *Rhodotorula sp.* are unable to do this, resulting in torularhodin being the primary end product.⁴¹ While the function of the carotenoids is not fully understood, in *Rhodotorula mucilaginosa*, torularhodin has been shown to protect the yeast from light exposure.⁷¹

Considerable attention has also focused on the production of lipids from this genus of yeast. The red yeast *Rhodotorula glutinis* has long been known for its high lipid content, being identified in the 1940s for its oleaginous properties. In 1949, oil from *R. glutinis* was described as being comparable to olive oil on the basis of its physical properties.²¹ *R. glutinis* is able to accumulate lipid up to 72% of its dry weight when glucose is used as the carbon source, but *R. glutinis* has also been shown to grow on a variety of carbon sources including hexose and pentose sugars,⁵⁷ and waste streams such as crude glycerol,⁷² thin stillage⁷³ and waste whey.⁷⁰ It has also been demonstrated that the biomass production of *R. glutinis* may be as high as 185 g/L when grown in a fed-batch culture with glucose as the substrate.⁷⁴ The lipid accumulation within *R. glutinis* is however strain-specific. For example, *R. glutinis* UCDFST 06-542 produced three times more lipid than *R. glutinis* UCDFST 50-309 when cultured under the same conditions.⁷⁵ Typically, the lipid profile obtained

from *R. glutinis* contains palmitic acid (16:0), oleic acid (18:1) and linoleic acid (18:2) as the main lipids. *R. glutinis* has also attracted research attention due to the fact that it can synthesise carotenoids.⁷⁶⁻⁷⁷ The combined characteristics offered by *R. glutinis* including the ability to produce oil from a wide variety of substrates, conferred resistance to lignocellulose degradation compounds, a suitable lipid profile, and the potential for high-value by-products, offers tremendous industrial potential for this yeast species, and hence has been selected as the organism of choice for this thesis.

While not as thoroughly investigated as *R. glutinis*, *R. minuta* has also been shown to be of interest for a variety of industrial applications. For example, *R. minuta* has been used on several occasions as a whole-cell catalyst for the enantioselective bioreduction of 1,2-diketones⁷⁸ and dialkyl oxoglucurates.⁷⁹ Isobutene production from *R. minuta* has also been reported, with L-leucine thought to be the precursor of the alkene.⁸⁰ To date, there are only two publications examining lipid production from *R. minuta*.^{46,81} Pan *et al.* reported a biomass yield of 8.1 g/L with a lipid content of 24.6% (w/w),⁴⁶ whereas Saxena *et al.* reported a maximum lipid yield of 48% (w/w) with the alkyl chain of the lipids in the C₇ – C₁₈ range.⁸¹ Very few yeast are known to produce medium chain lipids (C₇ – C₁₄) without genetic modification and as such, makes the industrial significance of this yeast an exciting possibility. *R. minuta* is also commonly found growing on the skin of grapes and has been demonstrated to be an active biocide-producing yeast.⁶⁰ In order to survive on grape skins, the yeast must be able to tolerate harsh acidic conditions and high osmotic pressure, thus it has the potential to be a highly suitable organism for biotechnological application. It is however currently unknown how *R. minuta* would behave in an industrial biotechnology system. *R. minuta* was therefore chosen as a second yeast species for research in this thesis.

1.6.3 Industrial potential of oleaginous yeast

Commercial production of SCO has been shown to be feasible if given an appropriate market and process. Current successful commercial ventures have only involved high-value speciality chemicals such as the antioxidant, astaxanthin from *Phaffia rhodozyma*.⁶⁹ This is due to the high costs surrounding the production of

yeast oil. In 2008, Ratledge and Cohen estimated that the cost of yeast oil was US \$3000 per tonne,³⁵ which was twice the cost of the equivalent plant oils. More recently, Huang *et al.*, calculated the cost of SCO production from yeast grown on lignocellulosic waste in China to be 7500 RNB (US \$1230) per tonne.⁸² Strain and process improvement is therefore essential to make the production of microbial commodity oleochemicals economically feasible. Improvements surrounding pretreatment and hydrolysis, improved utilisation of carbohydrates in lignocellulosic hydrolysates, faster microbial growth, increased oil accumulation, improved harvesting technologies and upgrading of spent yeast biomass into valuable co-products could all result in an economically-viable process.²¹

1.6.3.1 Feedstocks for yeast cultivation

For heterotrophic SCO production, a carbohydrate source is required. Due to the relatively low value of the oil-based product, the feedstock must be as cheap as possible to enable economic viability. Highly refined sugars such as glucose cost around US \$550/tonne and thus are too expensive for industrial application. Sugar-rich plant crops such as sugar cane or sugar beet can be used, but this is food-competitive which introduces questions surrounding the sustainability of the feedstock. Due to much of the criticism surrounding *first generation* feedstocks, it is essential that the oil produced is not food competitive. Many efforts have therefore focused on using low-cost waste materials as a growth medium for SCO production. Waste substrates have included, amongst others, banana juice, glycerol, molasses, whey, as well as wastewater from monosodium glutamate and olive oil mill processing.⁸² More recently, household food waste has also attracted attention as a waste feedstock for lipid production.⁸³⁻⁸⁴ *Second-generation* lignocellulosic (plant material) feedstocks such as switchgrass, sugarcane bagasse and wheat straw have also attracted interest due to the high carbohydrate content contained within the cellulose and hemicellulose polysaccharides.⁸⁵ However, because yeast lack a significant cellulolytic activity, the lignocellulosic material requires pretreatment and hydrolysis to release significant quantities of monomeric sugars. A number of biological, physical, chemical and physico-chemical pretreatment methods have been developed to extract soluble sugars from lignocellulosic biomass.²¹ The

pretreatment regime must be tailored towards the lignocellulose feedstock as well as to the target yeast strain, as different products are generated from each regime. Once the pretreatment has opened-up the structure of the polysaccharides and degraded the crystallinity of the cellulose fibrils, enzymes such as cellulases can be added to cleave the polysaccharides into oligo-, di- and mono-saccharides. The hydrolysis of lignocellulosic material, along with the waste feedstocks for SCO production is discussed in more detail in Chapter 5.

1.6.4 Lipid accumulation in yeast

Environmental growth conditions play a large part in the amount of lipid obtained. For example, factors such as pH, C/N ratio, oxygen, temperature, and concentration of trace elements and salts have been reported to have an effect on the lipid accumulation in yeast.^{36,86} While some species of yeast, such as *Cryptococcus terricola* can produce intracellular lipid continuously,²¹ most species produce lipid as a stress response induced by environmental change.²¹ Nutrient limitation has been widely investigated as a route to enhance lipid productivity.¹⁷ Most commonly, nitrogen limitation has been used as the trigger for enhanced lipid biosynthesis, however other substrate limitations have included sulfur,⁸⁷ phosphorous⁸⁸ and microelements such as zinc, iron or magnesium. In the case of nitrogen limitation, cell division, as well as protein and nucleic acid synthesis all cease once the nitrogen has been depleted from the culture medium. Meanwhile, if there is an excess of organic carbon such as glucose in the culture medium, this can be assimilated by the cells and converted into storage lipids, which leads to the accumulation of lipid.²⁰

Due to the taxonomic diversity of oleaginous yeast, it is quite possible that the ability to accumulate intracellular lipid bodies may have evolved multiple times independently, and thus there may be multiple mechanisms for lipid production.²¹ Presented here is the most commonly understood mechanism for lipid biosynthesis in yeast, based on work undertaken with *S. cerevisiae*. There are however a number of key distinctions between oleaginous and non-oleaginous yeast species. For example, oleaginous microorganisms produce significantly higher quantities of acetyl-CoA compared to non-oleaginous microorganisms.¹⁷

1.6.4.1 Production of acetyl Co-A from sugars

Under steady-state conditions, when glucose is used as the energy source, glucose enters the glycolysis pathway where it is converted into pyruvate through a multistep process yielding two molecules of pyruvate per glucose molecule. Pyruvate can then enter the mitochondrion and the tricarboxylic acid (TCA) cycle through its carboxylation by pyruvate carboxylase (TC) to oxaloacetate or the conversion to acetyl-CoA by pyruvate dehydrogenase (PDH). This leads to the accumulation of citrate within the mitochondrion, which at excess levels is exported from the mitochondria into the cytosol by the tricarboxylate carrier (TIC) in exchange with malate. The citrate is then cleaved by ATP-citrate lyase in the cytosol to yield oxaloacetate and acetyl-CoA (Figure 1.6).²²

1.6.4.2 Limitation-induced acetyl-CoA synthesis

Upon nitrogen exhaustion, the intracellular concentration of AMP (adenosine monophosphate) rapidly decreases as the AMP is converted into IMP (inosine-mono-phosphate) and NH_4^+ by AMP-desaminase. The release of NH_4^+ ions provides a temporary nitrogen source for DNA replication and protein synthesis.⁸⁹ AMP allosterically activates the mitochondrial isocitrate dehydrogenase, which is responsible for the conversion of isocitric acid to α -ketoglutaric acid, and as such, a decrease in AMP leads to intracellular accumulation of isocitric acid. Isocitric acid is found in equilibrium with citrate. These elevated levels of citrate within the mitochondrion result in its export into the cytoplasm in exchange with malate where it is cleaved into acetyl-CoA (Figure 1.6).¹⁷

1.6.4.3 ATP-citrate lyase (ATP-CL) and fatty acid biosynthesis

ATP-citrate lyase is a multi-enzyme complex found only in oleaginous microbial strains, and catalyses the formation of acetyl-CoA and oxaloacetate from citrate. The presence of ATP-CL can be used as a biological marker for oleaginity as it is also present in oleaginous plants and higher animals.²¹ In microorganisms where ATP-CL is not present, the elevated levels of intracellular citric acid caused by nitrogen limitation is generally excreted from the cell into the culture medium. This method of citric acid production has been observed in *Aspergillus niger* and

*Candida sp.*⁹⁰⁻⁹¹ This has led to yeast being classified as either lipid-accumulating or citric acid-producing.

In the oleaginous strains the acetyl-CoA continues in the pathway of lipogenesis, whereas the remaining oxaloacetate in the cytosol is converted to pyruvate, via malate by a malic enzyme, which can then re-enter the TCA cycle and generate NADPH. The NADPH is essential for the elongation of the alkyl chain of the fatty acid, and thus the malic enzyme controls the level of lipid accumulation.²² Similarly to ATP-CL, the malic enzyme is key to providing the oleaginicacy of a microorganism. For example, over expression of malic enzyme in *E. coli* in the presence of malate resulted in a four-fold increase in intracellular lipids.⁹²

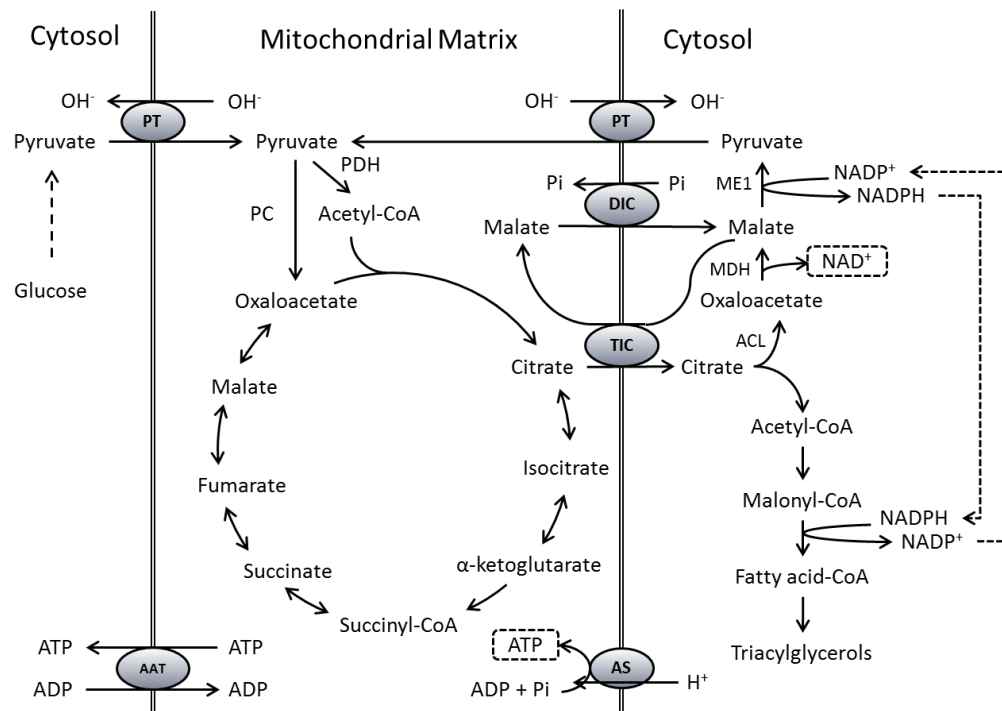


Figure 1.6: Triacylglyceride biosynthesis pathway from glucose to triacylglycerols in oleaginous yeast. AAT: ATP-ADP translocase; ACL: ATP-citrate lyase; AS: ATP synthase; DIC: dicarboxylate carrier; MDH: malate dehydrogenase; ME1: malic enzyme; PC: pyruvate carboxylase; PDH: pyruvate dehydrogenase; PT: pyruvate transporter; TIC: tricarboxylate carrier (adapted from Guay *et al.* and Ratledge).²⁻³

Once acetyl-CoA has been generated in the cytoplasm of the cell, it is carboxylated to form malonyl-CoA in an irreversible reaction by the enzyme, acetyl-CoA-carboxylase (ACC), requiring ATP and HCO_3^- . From this point onwards, the lipid

biosynthesis pathway is essentially a reduction of malonyl-CoA units to form the alkyl chain of the triacylglyceride by a multi-enzyme complex known as fatty-acid synthase (FAS) within the cytoplasm.⁹³ The FAS yields C₁₆ fatty acid molecules, which are released from FAS by a thioesterase. These free fatty acids are activated to Coenzyme A to yield palmitoyl-CoA, which are often further elongated to stearoyl-CoA (C₁₈). These are then esterified to glycerol by the α -glycerol phosphate acylation pathway (Figure 1.7).

In the lipid biosynthesis pathway, the activated acyl-CoA units are subsequently used for the acylation of glycerol-3-phosphate (3-G-P). Acylation firstly occurs at the sn-1 position, by G-3-P acyltransferase, to produce lysophosphatic acid before a second acylation at the sn-2 position to yield phosphatic acid. Following dephosphorylation by phosphatic acid phosphohydrolase, and subsequent acylation by diacylglycerol acyltransferase, the triacylglyceride is produced.¹⁷ This final enzymatic step has been established as an important rate limiting step in lipid accumulation since its overexpression in *S. cerevisiae* led to a 3–9-fold increase in triacylglyceride production.⁹⁴ Similarly, overexpression of thioesterases can deregulate the fatty acid synthase resulting in the over-accumulation of fatty acids.⁹⁵

While triacylglycerides are the major component of neutral lipids within SCOs, the intermediates of this pathway may be hydrolysed to produce di- and mono-glycerides, and occasionally free fatty acids. However, high concentrations of these intermediates within the cell are often indicative of lipid degradation rather than formation.⁹⁶

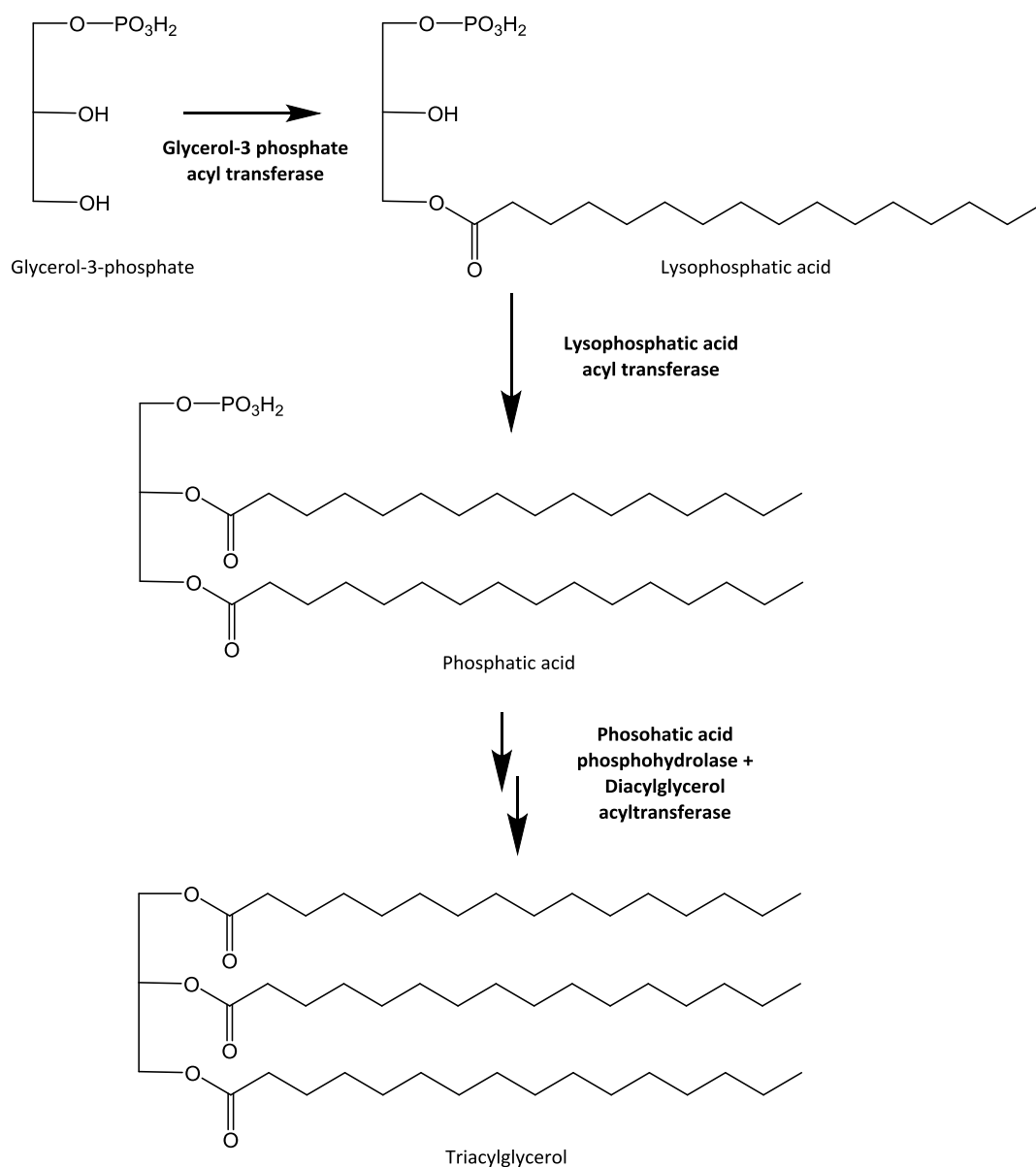


Figure 1.7: Schematic of intracellular triacylglyceride synthesis via the α -glycerol phosphate acylation pathway (adapted from Papanikolaou and Aggelis).¹⁷ Activated acyl-CoA units are used for the acylation of glycerol-3-phosphate at the sn-1 position by glycerol-3-phosphate acyl transferase to yield lysophosphatic acid. A second acylation occurs at the sn-2 position by lysophosphatic acid acyl transferase to produce phosphatic acid. Dephosphorylation by phosphatic acid phosphohydrolase, and subsequent acylation by diacylglycerol acyltransferase produces the triacylglycerol.

1.6.4.4 Chain length and degree of saturation

Fatty acid elongation and desaturation occur in the microsomal membrane. Desaturation is initiated by the introduction of a double bond at the $\Delta 9$ position of saturated fatty acids by a $\Delta 9$ -desaturase. Most yeast contain this desaturase, which is capable of producing palmitoleic ($C_{16:1}$) and oleic ($C_{18:1}$) acids. Some yeast also

contain two further desaturase enzymes, namely Δ^{12} -desaturase and Δ^{15} -desaturase which can introduce a second and third double bond, respectively, however the activity of these enzymes is species-specific. For example, the yeasts *Candida boidinni*, *Lipomyces starkeyi*, *Cryptococcus albidus* and *Zygosaccharomyces rouxii* can only produce linoleic acid as an unsaturated lipid.⁹⁷ When produced, linolenic is only produced in small quantities (approx. 1%) and has been observed in the yeast *Yarrowia lipolytica* and *Trichosporon pullulans*.⁹⁸

Interestingly, the fatty acid composition of the triacylglyceride is not randomly distributed on the glycerol backbone, but they are stereospecifically orientated. Greater than 65% of the FAs at the sn-1 position are commonly 16:0 and 18:1, whereas 18:1, 18:2 and 18:3 are most commonly found at the sn-2 position. The greatest variation in the FA localisation is found at the sn-3 position, which tend to be composed of 16:0, 18:2 and the greatest proportion of 18:3 in the triacylglyceride.⁹⁹ The fatty acid distribution in yeast tends to the type SUS and SUU, when S and U represent saturated and unsaturated fatty acids, respectively. The lack of saturated fatty acids in the sn-2 position of the glycerol backbone is also observed in most plant oils.

1.6.5 Carbon stoichiometry and lipid co-efficient

One of the most important metrics in the industrial production of lipid from oleaginous yeasts is the lipid co-efficient. This is the percentage of lipid produced per unit of sugar. For oleaginous yeast cultured on glucose, the maximum lipid yield is 22.4%. If all the glucose were directed towards lipid biosynthesis, the maximum stoichiometric yield would be 33% (w/w), however, some of the glucose is also used for cellular metabolism and biomass production. As such, 22% (w/w) has been proposed as the maximum productivity of oil from glucose.³⁵ This means that 5 tonnes of sugar is required for the production of 1 tonne of oil. As the carbon source is also used for the synthesis of the cell biomass that in turn holds the oil, there is a fine balance between oil production and the production of oil-free biomass. Ratledge *et. al.* suggested that a lipid content of 40% (w/w) is probably the optimum value to maximise the overall biomass yield,³⁵ with anything over this

value reflecting a reduction in cellular biomass rather than an increase in cellular lipid.

1.7 Extraction of oils from yeast and microalgae

Following the growth of the yeast cells and accumulation of the lipid, the yeast biomass first has to be harvested or separated from the culture medium. This step is often energy-intensive due to the large quantities of water that need to be removed and thus this step can contribute 20-30% of the total biomass production costs.²⁶ Common harvesting methods include sedimentation, centrifugation and ultra-filtration. Flocculation can be also used to aggregate cells. Once the yeast cells have been removed from the culture medium, the oil can be extracted from the cell. Whilst extraction techniques for yeast and algae are very similar, harsher conditions are required for algae due to the relative resilience of the cell. Generally, extraction methods include mechanical, organic solvent or supercritical fluid extraction.¹⁰⁰ The process of oil extraction from the cell is discussed in more detail in Chapter 6.

1.8 Industrially relevant products from oleaginous yeasts

1.8.1 Value of oil-free biomass

Current business models for SCO production view the oil as a single, valorised product.²¹ As such, following extraction of the oil, a considerable amount of residual, spent biomass will remain, which could be as high as 30% (w/w) of the starting substrate (assuming 100 g glucose produces 50 g biomass with 40% (w/w) lipid). In order for SCOs to be economically viable, the resulting oil should be viewed as one of several high-value products. For example, in the oilseed business models, co-products such as lecithin, sterols and texturised protein are produced alongside the oil.²¹ The remaining yeast biomass, while relatively high in protein, will also be rich in nucleic acids which are not desirable for human consumption as it can cause gout and kidney stones. In all likelihood, the biomass would be most suitable as an animal or fish feed supplement, however the method of extraction would need to be tailored for this application (e.g. no chlorinated solvents). The biomass could also be utilised as an energy source, either through direct

combustion by a combined heat and power (CHP) plant, or through anaerobic digestion into methane.³⁵ Either way, the value of this biomass is relatively low, especially in comparison to soybean meal.

1.8.2 Lipid derived road transport biofuels

In 2011, the UK's total final energy consumption was 147 million tonnes of oil equivalent (mtoe), of which 37.5% was accounted for by the transport sector.¹⁰¹ 97% of the energy used by the transportation sector was derived from petroleum (Figure 1.8). The transport sector is therefore one of the major anthropogenic sources of CO₂, responsible for creating 24% of the UK total greenhouse gas (GHG) emissions in 2009.¹⁰²

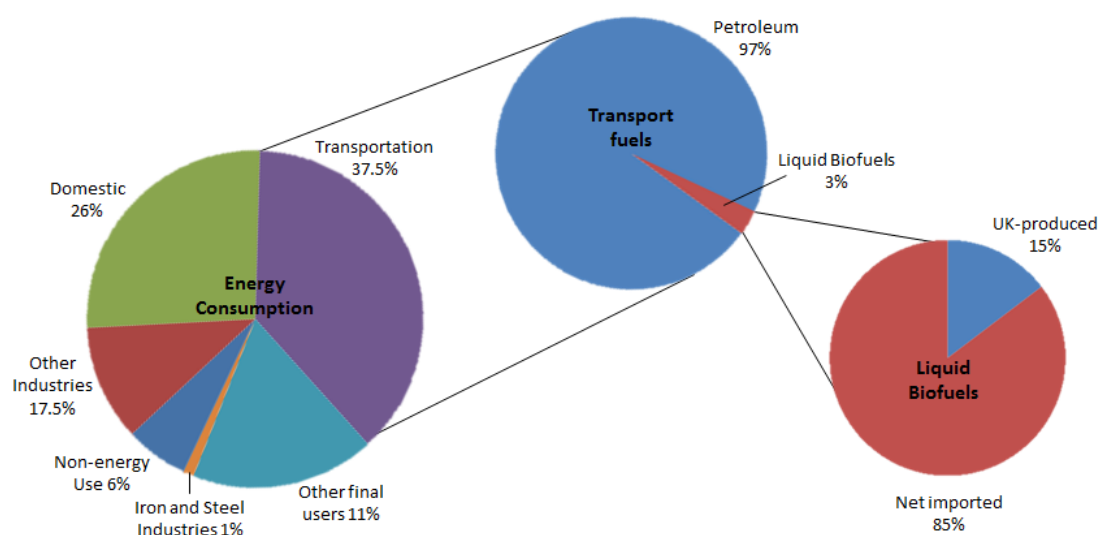


Figure 1.8: Net energy consumption in the UK. Data taken from the Department of Energy and Climate Change, *Digest of UK Energy Statistics*.¹⁰¹ The transportation sector accounts for 37.5% of the UK's energy consumption, with 97% of the transport fuels derived from petroleum. Of the 3% of biofuels that are used, only 15% are produced in the UK with 85% imported.

In the long term, ultra-low emission vehicles which include fully electric, plug-in hybrid, hydrogen and fuel cell powered cars offer an alternative to light-duty vehicles. However, the use of liquid fuels from renewable sources is an important transitional technology that is compatible with the current distribution network and offers a key feedstock for the production of heavy duty and aviation fuels.

The two dominant global biofuels are biodiesel and bioethanol, which can be blended with mineral diesel and petrol, respectively, with little or no modification to the vehicle engine. Due to the type of feedstocks and tax regimes available, bioethanol production is largely geographically oriented within the United States and South America, whereas in the EU, biodiesel is the most commonly produced biofuel. In recent years, the use of liquid biofuels in the transportation sector has shown considerable growth. In the UK, approximately 3% (v/v) of transportation fuel is derived from renewable feedstocks, driven mostly by an array of government policies focused on enhancing energy security, mitigation of GHG emissions, protecting fuel consumers against price increases and promoting economic growth in rural areas.¹⁰²

The vast majority of biodiesel (95%) is produced from *first-generation* edible feedstocks, such as rapeseed (84%), sunflower (13%), palm oil (1%), soybean and animal fats (2%).¹⁰³ These fuels have attracted considerable public criticism, mainly due to concerns over land usage and food security. This has raised pertinent questions on the sustainability of biofuels, as the demand for biofuels could have devastating environmental and social repercussions due to the pressure on natural resources.¹⁰⁴ Because of this, in June 2014, biofuels made from food crops were capped at 7% for road transport.¹⁰⁵ Increasing public pressure coupled with the cost of these first generation feedstocks has driven the development of *second generation* sources, produced from non-food feedstocks. These include plant oils such as jatropha¹⁰⁶ and jojoba¹⁰⁷ which can be grown on marginal land, as well as waste oils including cooking oils¹⁰⁸ or agricultural residues such as animal fats.¹⁸ Several companies produce biofuels from these feedstocks including Argent Energy (UK) who produce biodiesel from waste cooking oils, fats from the food industry and fats that accumulate in the sewer network.¹⁰⁹⁻¹¹⁰

1.8.2.1 Biodiesel production

Triacylglycerides can be used without modification as a diesel substitute. However, the high viscosity and low volatility lead to engine coking, carbon deposition and gelling of the lubricating oil.⁴ To convert the triacylglycerides into a suitable fuel, a variety of methods such as pyrolysis and cracking have been suggested,¹⁰³ but

transesterification of the triacylglycerides has been the most widely used method to date.¹⁵ Transesterification decreases the viscosity to around 15% of the parent oil, depending on the lipid profile. For transesterification, the triacylglycerides are reacted with a short chain alcohol (such as methanol) and a catalyst to form fatty acid alkyl esters (FAAEs). The reaction proceeds stepwise, in which the triacylglycerides are first converted to diglycerides, then to monoglycerides and finally to glycerol and FAAEs. The reaction is in equilibrium, with 3 moles of alcohol needed to convert each mole of triacylglyceride. This yields 3 moles of FAAE (biodiesel) and 1 mole of glycerol (Figure 1.9). In order to shift the reaction in favour of FAAE production, the alcohol is generally added in a large excess to produce alkyl esters of greater than 98% on a weight basis.²⁴

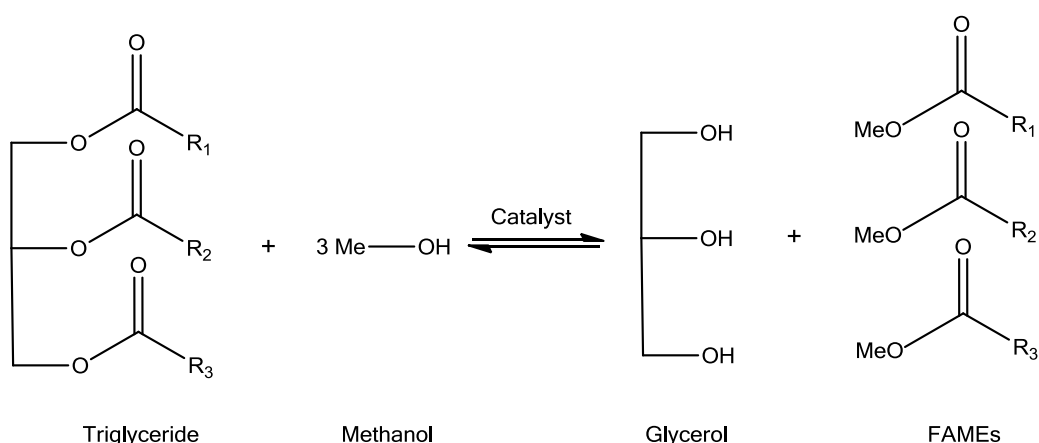


Figure 1.9: Transesterification of a triacylglyceride into biodiesel (FAMES) with methanol. One mole of triglyceride is reacted with three moles of a short chain alcohol (in this case methanol), in the presence of a catalyst (e.g. sulphuric acid) to yield one mole of glycerol and three moles of Fatty Acid Methyl Ester (FAMES). The reaction proceeds at equilibrium, but the alcohol is often added in excess to favour the formation of glycerol and FAME.

The transesterification is generally base catalysed, commonly by potassium or sodium hydroxide, though strong acids such as sulphuric acid also have a moderate activity. Enzymatic transesterification using lipases have also been investigated due to their high selectivity, mild operating conditions and ability to transesterify triacylglycerides with a high free fatty acid (FFA) concentration. However, this latter method is not currently commercially feasible due to the high cost and

poisoning of the enzyme at the high concentrations of methanol necessary for high conversion yields.¹¹¹

Once transesterified, the biodiesel can be used as a neat fuel (B100), but it is more commonly used in a blend ratio with petrodiesel. In the USA, biodiesel is often used as a B20 blend (20% biodiesel, 80% petrodiesel), whereas in the European Union this value often decreases to B5-7 to comply with governmental regulations.¹¹² While the use of biodiesel produces less sulphurous, hydrocarbon, and particulate emissions, the poor low temperature properties, increased NO_x emissions and low oxidative stability limit the applicability.¹¹²

1.8.2.2 Biodiesel fuel properties

The lipid profile of the biodiesel oil feedstock has a significant effect on the physical properties of the resulting fuel, and as such it is important to align the oleaginous yeast with the intended application. Other lipid-soluble components synthesised by yeast can also have detrimental effects on the fuel properties. These include terpenes, hydrocarbons, sterols and phospholipids. Sterol glucosides, which have limited solubility in biodiesel, have a melting point of greater than 240 °C, and can precipitate when stored at low temperatures.¹¹³⁻¹¹⁴ These precipitates can lead to fuel filter plugging if present in the fuel. The chain length, the degree of unsaturation and branching of the lipid all influence the physical properties of the oil. Therefore, the biodiesel must comply with regulatory standards, most notably ASTM 6751-02 and EN 14-214 in the US and EU, respectively (Table 1.3).

Table 1.3: Current selected specifications in biodiesel standards (ASTM D6751 in the United States; EN 14-214 in Europe) caused by the fatty acid composition and heteroelements.¹¹⁵

Specification	ASTM D6751	EN 14-214
Cetane Number	47 (-) min.	51 (-) min.
Kinematic viscosity	1.9-6.0 mm ² s ⁻¹	3.5-5.0 mm ² s ⁻¹
Oxidative stability ^a	3 h min.	6 h min.
Cloud point	Report	-
Cold filter plugging point	-	^b
Cold soak filtration test	360 s max.	-
Sulfur	0.0015 mass% (15ppm) max for ULSD 0.05 mass% for 500 ppm sulphur diesel	10.0 mg kg ⁻¹ max.
Na + K combined	5.0 (µg g ⁻¹) max.	5.0 µg g ⁻¹ max.
Ca + Mg	5.0 (µg g ⁻¹) max.	5.0 µg g ⁻¹ max.

^a Rancimat test per standard EN 14-214. ^b Depends on geographic location and time of year. Maximum (max.) and minimum (min.) values.

The overall composition of the fatty acid profile of the fuel feedstock affects the cetane number, kinematic viscosity, oxidative stability and cold flow properties of the fuel.¹¹⁶⁻¹¹⁸ For example, feedstocks that are high in saturated esters have poor low temperature properties, a high viscosity but suitably high cetane numbers. Conversely, a feedstock rich in polyunsaturates have low melting points and low viscosity but also have severely reduced cetane numbers (Table 1.4).⁴

Table 1.4: Properties of the fatty acid methyl esters found in yeast biodiesel. Data adapted from Knothe *et al.* and Schonborn *et al.*^{4, 119}

Property	Fatty acid methyl ester				
	16:0	18:0	18:1	18:2	18:3
Cetane number	81	89	62	42	22.7
Kinematic viscosity (40°C; mm ² /s)	4.38	5.85	4.51	3.65	3.14
Oxidative stability (110°C; h)	>24	>24	2.79	0.94	0.00
Density (g/cm ³) 15°C	0.867	0.868	0.877	0.890	0.902
40°C	0.849	-	0.859	0.872	0.883
Melting point (°C)	28.5	37.7	-20.2	-43.1	-45.5

Plant-based oils, currently used for the production of biodiesel (e.g. soybean or rapeseed) contain primarily C₁₆ and C₁₈ fatty acids, with varying degrees of unsaturation.¹²⁰ This is also similar for yeast biodiesel, but the latter lacks the polyunsaturated lipids found in plant oils (e.g. linolenic acid, 18:3). While the greater quantity of polyunsaturated lipids in plant oils vastly improves the low temperature properties of the fuels, it decreases the cetane value of the fuel and has poor oxidative stability.⁴ Because of this, lipids with four or more double bonds are limited to a maximum of 1 mol.%, and more specifically, the linolenic acid (18:3) content of the fuel is limited to 12 mol.% in the EU, in accordance with the biodiesel standard EN 14-214. Additionally, increasing the amount of unsaturated fatty acids also increases the NO_x emissions from the fuel.¹²¹ This is because the cetane number of the fuel decreases as the degree of unsaturation increases.¹²² This in turn increases the ignition delay and thus more fuel is injected and mixed with air before ignition occurs, favouring NO_x formation.¹²³ In contrast, the greater amount of oleic acid ester (18:1) generally found in yeast oils will be advantageous for oxidative stability without compromising on the cold flow properties.¹¹⁷ It was also demonstrated that soybean oil high in oleic acid produced less NO_x emissions

when compared to regular soybean biodiesel.¹²⁴ The relationship between the lipid structure and the effect on the resulting biodiesel is summarised in Table 1.5.

Table 1.5: The relationship between structure and biodiesel performance parameters.⁷⁵

	Cetane number (ignition quality: greater is better)	Melting point (lower is better)	Oxidative stability (more stable is better)	Kinematic viscosity (less viscous is better)	Heat of combustion (greater is better)
Chain length	Longer is better	Shorter is better	NR	Shorter is better	Longer is better
Degree of unsaturation	Saturated is better	Unsaturated is better	Saturated is better	Unsaturated is better	NR
Branching	NR	Branching is better	NR	NR	NR

NR = Not Relevant

1.8.2.3 Fuel properties of microbial biodiesel

It is essential that the specifications for biodiesel standards are met regardless of the feedstock used. While there are several reports that have examined the fuel properties of biodiesel from microalgae, there are very few studies that discuss the properties of biodiesel produced from oleaginous yeast. In general, the lipid profile of algal oils is more complex compared to plant and yeast oils, with the alkyl chain ranging in length of C₁₂ – C₂₂. Microalgal lipids are also highly polyunsaturated, with eicosapentaenoic acid (20:5) and docosahexanoic acid (22:6) commonly found.²⁶ This high level of polyunsaturation will have negative effects of the oxidative stability of the fuel as well as increasing the likelihood of the fuel polymerising in the engine. Therefore, if algal oils were to be used as a biodiesel feedstock at blend levels greater than a few percent, the feedstock would need to be partially hydrogenated to improve its physical properties. Furthermore, algal oil contains the lipid-soluble Mg-containing chlorophyll complex. While only present in small quantities, if used at scale the presence of magnesium in the fuel could have detrimental effects on the engine performance and longevity due to inorganic ash formed in the combustion process.¹²⁵ ASTM specifications therefore require that the maximum content of calcium and magnesium combined should not exceed 5 ppm.

Wahlen *et al.* compared the fuel characteristics of biodiesel from three microbial sources: the yeast, *Cryptococcus curvatus*; the bacteria, *Rhodococcus opacus*; and

the green microalgae, *Chaetoceros gracilis*. These fuels were compared to petroleum diesel and soybean-derived commercial biodiesel.¹²⁶ The fuel properties were examined and then the emissions and performance of the fuels were tested in a 2-cylinder research engine via a controlled engine test procedure. The lipid profile of the biodiesels tested are shown in Table 1.6 (lipid under 0.6% (w/w) have been excluded).

Table 1.6: Fatty acid profile of the biodiesel fuels tested in an engine.¹²⁶

	FAME [†] (% of total fatty acids)										
	14:0	15:0	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	20:5
Soybean	-	-	11.0	-	-	-	3.9	23.7	53.9	6.8	-
Microalgae	10.3	-	26.8	29.9	6.9	8.9	0.5	3.7	2.2	4.1	5.8
Yeast	-	-	15.3	0.2	-	-	18.0	59.8	5.3	0.3	-
Bacteria	1.9	4.4	33.9	8.9	-	-	3.0	23.9	-	-	-

[†]Fatty acid methyl ester. The oil species tested were: Soybean (*Glycine max*); Microalgae (*Chaetoceros gracilis*); Yeast (*Cryptococcus gracilis*); Bacteria (*Rhodococcus opacus*).

The yeast biodiesel was composed only of C₁₆ and C₁₈ fatty acids, containing 60% mono- and 6% poly-unsaturates. The kinematic viscosity of the yeast biodiesel measured 4.5 mm² s⁻¹, within the range for both ASTM D6751 and EN 14-214 specifications. Both the energy density and heating value of the yeast-based fuel were similar to the commercial soybean biodiesel, whereas the biodiesel cetane index (using the density of the fatty acids to predict a cetane value) for the yeast biodiesel was considerably higher than soybean biodiesel at 67 (-). The yeast biodiesel cetane number is therefore comfortably above the 51 (-) minimum for EN 14-214 specification (Table 1.3). Power output from the yeast oil was 93% and 96% of the output observed for petroleum diesel and soybean biodiesel, respectively. The yeast biodiesel fuel also produced similar levels of CO₂ emissions to petroleum diesel, but produced less than half of the CO emissions than petroleum diesel. Interestingly, the levels of NO_x emissions were 37% higher for the yeast biodiesel and 9% higher for the soybean oil compared to petroleum diesel. The bacterial biodiesel showed the largest increase in NO_x output, being 81% higher than that observed for diesel. Increases in NO_x emissions are known to be due to an increasing degree of polyunsaturation present in the oil or due to increasing chain length.¹²¹ The shorter chain lengths found in the microalgal oil were believed to be responsible for the 24% reduction in NO_x emissions for microalgal oil compared to

that of petroleum diesel. Aside from the NO_x emissions, all the microbial oils tested were shown to have similar physical properties and engine performance to the soybean biodiesel, suggesting that SCOs can be effective feedstocks to displace both petroleum diesel and plant biodiesel.

1.8.3 Lipid derived aviation biofuels

Until the development of the jet turbine engine in the 1940s, aircraft engines were run on similar fuel blends to that used in motor vehicles. The development of the turbine engine during World War II led to the need for a fuel with a high flash point but low freezing point. As such, a separate fraction of crude oil was developed to fulfil this requirement. The term jet fuel covers all fuel used in gas-turbine powered aircraft, and is often referred to as kerosene or aviation turbine fuel. Today, around 16,000 jet aircraft operate globally, using around 200 million tonnes of kerosene per annum. The fuel accounts for 10-15% of airline operating costs.¹²⁷ The current release of CO₂ from the combustion of aviation kerosene accounts for 2% of total anthropogenic CO₂ emissions. However, due to the release of water vapour, sulphate and soot particles in the upper atmosphere, it has been estimated that the total impact of aviation on the climate is two to four times higher than the effects of the carbon dioxide emissions alone.¹²⁸ As such, the industry has set itself a target to half emissions by 2050, compared to 2005 levels.

Aviation kerosene is currently produced through the cracking and distillation of crude oil; the kerosene fraction being produced at 150-275 °C. It can consist of greater than 1000 different chemical compounds, but the main components are hydrocarbons, ranging between C₈–C₁₆ in length, containing both straight and branched isoparaffins and cycloparaffins (naphthenes). Up to 25% of the fuel is composed of aromatic units as well as trace amounts of sulphur-, oxygen- nitrogen-, and hetero-containing hydrocarbons.¹²⁹ These minor components play a large part in the fuel stability, lubricity, corrosivity and cooling characteristics.¹³⁰ Due to inconsistencies within the crude oil feedstock, aviation jet fuel produced from different feeds and processes have vastly different ratios of these components and as such there is a large variation in physical properties. All jet fuel has to comply with strict operational standards. The major legal specifications for commercial

aircraft are Jet A in the US and Jet A-1 used in the EU and globally. The standards are similar (Table 1.7) though for Jet A-1 the freezing point of the fuel is lower than Jet A (-47 °C and -40 °C, respectively) which makes it more suitable for long-haul flights, especially those on polar routes during the winter.¹³¹ Chemical additives, which can enhance or preserve the fuels physical properties are also added to the fuel and can consist of antioxidants, metal deactivators or biocides.

Table 1.7: Standard specifications for Jet A and Jet A-1 turbine fuels and comparative properties for HEFA* fuels.

Fuel Property	Units	Jet A [†]	Jet A-1 [‡]	HEFA [*]
Flash point	°C	Min. 38	Min 38.0	
Density at 15 °C	kg/m ³	Min. 775 Max. 840	Min 775.0 Max. 840.0	0.730
Freezing point	°C	Max. -40	Max. -47.0	-57
Total aromatics	% v/v	Max. 25	Max. 26.5	
Viscosity at -20 °C	mm ² /s	Max. 8	Max. 8.000	
Specific energy	MJ/kg	Min. 43.0	Min. 42.80	47.4

[†] ASTM D1655. Standard specification for aviation turbine fuels.

[‡] DEF STAN 91-91. British Ministry of Defence standard specification. Turbine fuel, kerosene type.¹³²

^{*} Hydroprocessed esters and fatty acids.

Currently, it is estimated that 7 million barrels of kerosene are used globally, and thus the challenge to create an alternative product on this scale is considerable.¹³³ Whilst other transportation sectors are expected to decrease their emissions by 2050, aviation emissions are increasing – doubling since 1990. Worldwide aviation is expected to grow by 5% annually up to 2050.¹³⁴ Consequently, UK aviation emissions are predicted to increase from 9% in 2005 to 29% in 2050.¹³⁵ Historically, fuel efficiency gains have been achieved by operational improvements (e.g. higher load factors, utilisation of larger aircraft) or technical progression (e.g. more efficient engines, lighter aircraft). While this is set to continue, the efficiency gains will not off-set the increase in aviation traffic and fuel consumption is predicted to increase by 3% annually.¹³⁴

Therefore, changing the fuel source is one of the few options available to the aviation industry in order to reduce its carbon footprint, due to the long life of commercial jet planes. A number of government initiatives have sought to increase activity in this area. Firstly, the renewable energy directive issued in 2009 set a mandatory target that 20% of energy should come from renewable energy sources

by 2020. In 2011, the European Commission, in partnership with several European airlines and biofuel producers, launched an initiative to speed-up the commercialisation of aviation biofuels in Europe. The *European Advanced Biofuels Flight path Initiative* set-out to achieve an annual production of two million tonnes of sustainably produced aviation biofuel by 2020.¹³⁶ Furthermore, in 2012, the European Union introduced legislation for the aviation sector to join the EU Emissions Trading Scheme (EU ETS), meaning that emissions will be capped to ensure that any increases in aviation emissions are offset by reductions elsewhere in the EU economy, or internationally. For aviation, emissions will be capped at 95% of historical emissions (the estimated average emissions for the years 2004–2006). The transition to biofuels is one of the few options available to aviation to be able to achieve the reduction in GHG emissions. This aims to allow the aviation industry to find cost-effective solutions for CO₂ reduction without significant governmental intervention. In the scheme biofuels have an emission factor of zero, whereas petroleum-derived jet fuel corresponds to 3.15 kg of CO₂ per kg of jet fuel.

Due to the global nature of the aviation fuelling infrastructure, the characteristics of airport fuelling systems (which tend to have a single storage and distribution for all vehicles) and the slow and costly replacement of aircraft fleet, the aviation community is focused on *drop-in* replacements that have been shown to be functionally identical to petroleum-derived jet fuel. The Jet A and Jet A-1 fuel specifications are extremely stringent, making it more challenging to produce an alternative aviation fuel than a road transport replacement.¹³⁷ The alternative jet fuel must be able to meet recognised fuel properties such as a high energy density, a freezing point below -47 °C and a low viscosity of below 8 mm² s⁻¹ at -20 °C (Table 1.7).¹³³ Beyond the specification, an alternative jet fuel must also be compatible with the existing infrastructure, including the fuel delivery system, components and demonstrate material compatibility. It must also be able to combust safely and reliably within extreme operating conditions. Over recent years there have been a number of commercial ventures to produce economically-viable *drop-in* replacements for petroleum-derived jet fuel.

1.8.3.1 Lipid derived fuels for the aviation sector by hydroprocessing

Due to the poor low temperature properties of biodiesel, the lipid must be hydroprocessed to remove the oxygen group before cracking and isomerisation to produce a suitable jet fuel (Figure 1.10).

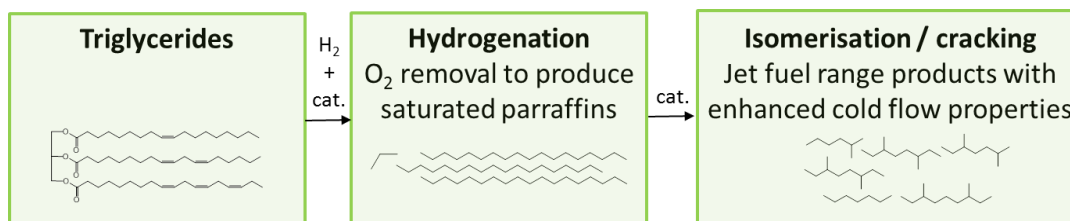


Figure 1.10: Representation of the production of hydroprocessed esters and fatty acid (HEFA) jet fuel via hydrogenation and isomerisation. H_2 = hydrogen; cat. = catalyst.

The process firstly involves hydrogenation to completely saturate the lipid followed by the catalytic removal of oxygen. Hydrogen demand for hydroprocessing of different feedstocks varies, due to the variation in saturation in the oil feedstock. Highly saturated oils such as palm oil or animal fats will require less hydrogen and therefore incur cost savings compared to highly polyunsaturated oils (such as those from microalgae). Following hydrogenation, the oils are then isomerised or cracked and isomerised to produce paraffins within the jet-fuel range that have improved low temperature properties compared to the feedstock. This process yields a jet-fuel containing predominantly branched paraffins that can be blended up to 50% (v/v) with petroleum-derived jet fuel.¹³⁸ Hydroprocessed esters and fatty acids (HEFA) jet fuels therefore tend to be shorter chained branched alkanes (C_7 - C_{16}). HEFA are a highly attractive fuel substitute due to the low aromatic and very low sulphur content. Neste Oil is currently operating several plants at full commercial scale using this process. Two, 190,000 tonnes/annum HEFA plants are operational in Finland, alongside an 800,000 tonnes/annum plant in Singapore.¹³⁹ This process (NExBTL) yields fuel fractions for both the road transport and aviation sectors. Recently, high yields (77%) of hydrocarbons suitable as a blending agent with jet-fuel (C_9 – C_{16}) have been produced through the catalytic conversion of algal oil using mesoporous zeolites in a single-step process.¹³⁷

Table 1.8: Chemical composition of jet fuel.¹³⁰

Composition (%)	Jet-A1	HEFA [†]
n-alkanes	19.6	~10
Iso-alkanes	29.9	~90
Monocyclic alkanes	20.3	Trace
Polycyclic alkanes	7.3	Trace
Alkyl benzenes	14.1	0
Other hydrocarbons	8.7	0

[†]Hydroprocessed esters and fatty acids.

1.8.3.2 Application of HEFA fuels in flight

Since 2008, a variety of demonstration and commercial flights have taken place using HEFA biofuels. These fuels have generally been produced from vegetable oil feedstocks such as jatropha, camelina and waste cooking oil, used at low blend levels. The fuel produced from these *second-generation* sources reduced the carbon footprint of the fuel by up to 80% relative to petroleum-derived jet fuel.¹⁴⁰ The world's first commercial flight using biofuels, a Boeing 737-800 operated by KLM Royal Dutch Airlines occurred in July 2011, with the UK's first commercial flight occurring in October 2011. Airbus Group (formerly known as EADS) were the first to demonstrate an aircraft flight using 100% algal-derived HEFA fuel (on one engine) at the ILA (International Luft- und Raumfahrttausstellung) air show in Berlin in June 2010,¹⁴¹ with the first commercial flight using 40% algal fuel taking place in November 2011 operated by Continental Airlines. In September 2012, the Australian biofuel company, Algae. Tec, signed a collaboration agreement with the German airline Lufthansa, for the construction of a large-scale pilot plant to produce aviation biofuels from microalgae.¹⁴² Since then, many other companies such as Solazyme, Dynamic fuels, Sapphire, Honeywell UOP, ARA, Chevron, Lummus global and JetBio have been developing lipid-derived HEFA jet fuel,¹⁴³⁻¹⁴⁵ thus demonstrating the potential of HEFA as a commercially-viable process. While yeast oil-jet fuel has not yet been attempted on an industrial scale, the technology surrounding the upgrading of this feedstock has been demonstrated repeatedly for similar feedstocks and thus offers high potential.

1.8.4 Alternative industrial uses of lipids

While the technology exists to convert lipids into various types of fuel, there are many concerns surrounding the economic viability of this route to produce bulk

equivalents of major commodity fuels. For example, the biodiesel fatty acids esters could be used as biodegradable lubricants for use in chainsaw oil, gearbox oils and hydraulic oils.¹⁴⁶ Indeed, many companies have changed their business strategy in the last few years in favour of a broader product portfolio, especially surrounding microalgal oils. For instance, Aurora Biofuels is now Aurora Microalgae, Solix Biofuels is now Solix Biosystems and Solazyme, once focused entirely on fuels now describes itself as a renewable oils company.¹⁴⁷ An alternative is to exploit the differing structures and thus properties of the oils that can be produced, and tailor the oil towards the higher-value applications. These include (1) palm oil; (2) cocoa butter; (3) γ -linolenic acid (18:3); and (4) polyunsaturated fatty acids (PUFAs).³

Palm oil is a saturate-rich oil extracted from the seed and mesocarp of the oil palm fruit, and it is used widely in food and personal care products. Due to the high proportion of saturated oils, it is mainly used in the production of surfactants. These include the non-ionic surfactants, alkyl polyglycoside and fatty alcohol ethoxylate, and the anionic surfactant fatty acid sulphate.¹⁴⁶ The production of palm oil has received substantial criticism in recent years, particularly due to large areas of rainforest in Malaysia and Indonesia being cleared for its cultivation. As such, companies that currently use significant amounts of palm oil in their products are seeking sustainable alternatives. In March 2010, Unilever formed a partnership with Solazyme to develop their tailored™ algal oils, to be used in its Brylcreem, Lux and Dove products.¹⁴⁸ Similarly, in 2014 Ecover launched its new range of sustainable cleaning products using algal-derived surfactants rather than palm oil.¹⁴⁹ Similarly to palm oil, cocoa butter is used in many food and cosmetic applications, though it has a slightly different lipid profile. Cocoa butter substitutes require approximately equal concentrations of steric acid (18:0), palmitic acid (16:0) and oleic acid (18:1). A microbial cocoa butter substitute was produced by inhibition of the $\Delta 9$ -desaturase in *Candida curvata*,¹⁵⁰⁻¹⁵¹ though the full-scale deployment is reliant on its economic competitiveness with terrestrial cocoa butter.

Another high value lipid derivative is γ -linolenic acid (18:3), which cannot be synthesised by the human body, but it has received significant attention from the homeopathic community due to the range of health benefits linked to its

consumption. Linolenic acids are found in fish oils, but concerns surrounding increased fish consumption, marine pollution and decreasing fish populations require an alternative, sustainable source.⁹⁸ One alternative is evening primrose oil which contains approximately 9% γ -linolenic acid, and has been sold as an over-the-counter supplement for the last 40 years.³ Linseed oil is also particularly distinctive for its large quantity of linolenic acid (~55%), but it is less common for human consumption due to its susceptibility to polymerise with exposure to air. The filamentous fungi, *Mucor circinelloides* has also been used for its production.²¹ In most yeast, linolenic acid is found in concentrations of approximately 1%, however expression of an ω -3 desaturase from *F. moniliforme* within *Y. lipolytica* yielded more than 28% of the total fatty acids as α -linolenic acid.¹⁵² Long-chain PUFAs are also of considerable interest, again due to the potential health benefits that they may provide as a dietary supplement. More specifically, arachidonic acid (20:4), eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) are actively being pursued as they are essential fatty acids found in brain tissue.⁹⁸ Alkyl chains of this length are not generally found in yeast, however they can be found in several species of microalgae.¹⁵³

1.8.4.1 Lipids as a feedstock for the chemical industry

Various types of polymers, including polyesters, polyurethanes, polyamides, epoxides and vinyl polymers can be produced from lipid feedstocks utilising different polymerisation reactions.¹⁹ For example, polyesters can be produced through the condensation of the hydroxyl group (-OH) of the fatty acids. More novel polymers based on dicarboxylic acids and reactions surrounding the double bond on the alkyl chain can also be produced from oils. Through ozonolysis, azelaic acid can be formed from which polyamides such as nylon 6,9 can be produced. Similarly, dimerization of fatty acids can yield polyamido amines such as epoxy curing agents as well as non-nylon polyamides. Epoxidation of the double bonds can produce polyols such as polyurethanes and linoleum. Epoxidised soybean oil can also be used as a plasticiser.¹⁴⁶

Palm, soybean, rapeseed, sunflower and animal fats have been used as feedstocks for polymer and lubricant applications.¹⁴⁶ These polymers generally have a positive

public perception and they can also display enhanced physical properties.¹⁹ Oils such as linseed oil and soybean oil, with a high degree of unsaturation have been used in oil-based paint surface-coating materials due to the solid film-like material that forms upon crosslinking with atmospheric oxygen molecules.¹⁴⁶ Polymerised linseed oil has also been used in linoleum manufacture. The variation in the fatty acid profile and their relative percentages will affect the polymeric properties. For example, polymers produced from an oil rich in oleic acid (18:1) resulted in a higher quantity of flexible side chains and thus a less compact polymer.¹⁵⁴ The degree of unsaturation within a particular oil feedstock can be compared using the iodine value of the oil, with a greater value conferring a greater degree of unsaturation (Table 1.9). Whereas a low iodine value is required for biodiesel replacements, high iodine values are often beneficial for polymer production.

Table 1.9: Iodine value of common vegetable oils.¹⁹

Oil	Iodine Value (g of I ₂ /100 g)
Castor (<i>Ricinus communis</i>)	102.2
Coconut (<i>Cocos nucifera</i>)	15.1
Corn/Maize (<i>Zea mays</i>)	123.5
Linseed (<i>Linum usitatissimum</i>)	180.0
Palm (<i>Elaeis guineensis</i>)	43.3
Soybean (<i>Glycine max</i>)	128.7
Sunflower (<i>Helianthus annuus</i>)	120.0

1.9 Summary

Consumer products derived from crude oil are present everywhere in modern society. Similarly, the use of petroleum as an energy source far outweighs the use of coal, natural gas, nuclear and renewable energy supplies.²³ The unsustainable consumption of petro-products, coupled with concerns surrounding the environment, energy security, and soaring oil prices have however directed policymakers and scientists towards other, renewable alternatives. In the near-term, *drop-in* replacements, compatible with current infrastructure and processes are one of the best options available in the transition away from fossil fuels. While oil-rich terrestrial crops such as rapeseed or palm oil offer a suitable alternative, it is becoming increasingly clear that fuels and chemicals produced from these feedstocks are causing large ecological damage, have a negative public image and

are in too small supply to meet demand. Therefore, oleaginous microorganisms such as yeast and microalgae which can produce up to 80% of their dry cell weight as oil are being actively researched as a promising source of lipids. While there is the tendency to group all species and strains of oleaginous yeast into a single entity of biotechnological significance, the yeast species should be carefully selected towards the target product. Oleaginous yeast, as a group, do however offer great potential for commercialising a variety of products. In order to achieve this, firstly, the lipid profile should be compatible with the desired application. Oils that are high in saturated fatty acids would make an ideal replacement for palm oil, used primarily in foodstuffs and cosmetics; whereas oils rich in monounsaturates, such as oleic acid (18:1) upon transesterification, would make an ideal replacement for diesel transport fuel. Conversely, highly polyunsaturated oils, such as linolenic acid (18:3), which is not suitable as a road transport fuel, but instead would be ideal as a healthcare supplement or for chemical upgrading within polymer applications. Regardless of the end application, the desired yeast should also confer suitable characteristics that enable the use of different hydrolysates, as well as offering high growth rates and lipid productivities.

1.10 Aims and objectives

The overarching aim of this thesis is to progress understanding into the production of commercially-viable yeast oil for fuel and commodity application. The yeast *Rhodotorula glutinis* was selected due to its high growth rate and lipid productivity, simple lipid profile and the ability to grow on both hexose and pentose sugars. It has also been shown to produce mainly oleic acid (18:1) and palmitic acid (16:0) which offer commercial potential for biodiesel and cosmetic applications, respectively. Similarly, the yeast *Rhodotorula minuta* has been shown to produce a lipid profile that is high in medium-chain lipids (C₇ - C₁₄) which offer the physical properties desired by the aviation fuel industry. However, the lipid profile of oleaginous yeast can be highly variable and sensitive to changes in the environmental growth conditions. To assess the extent to which the environmental growth conditions can affect the resulting oil, in **Chapter 3**, *R. glutinis* and *R. minuta* will be grown under a variety of different temperatures and C/N ratios, as designed

using a response surface methodology. **Chapter 3** will also explore the extent to which the lipid profile can be tailored towards a desired application, and how the microbial lipid can slot into current legislation.

In order to address some of the problems surrounding commercial viability, several different routes will be explored. Firstly, to achieve the highest possible efficiencies from a glucose feedstock, in **Chapter 4**, technologies adapted from the bioethanol research field will be studied to enhance glucose conversion to oil using *Rhodotorula sp.* Secondly, due to the large cost surrounding the feedstock for yeast growth, the transition to waste feedstocks will be explored in **Chapter 5**. This exploration will be achieved by growing *Rhodotorula sp.* on mixed-sugar media, as well as in the presence of common microbial inhibitors present in depolymerised feedstocks. Using real-life scenarios, *Rhodotorula sp.* will also be grown on two low cost substrates: depolymerised *Miscanthus* and waste food.

To reduce the cost surrounding the energy consumption of the extraction of the oil from the yeast cell, **Chapter 6** will use an energy return on investment methodology to assess the feasibility of microwave extraction for this application. Finally, the economics of the microbial oil route as a whole will be assessed. This will be achieved by producing an energy balance of the microbial oil process from food waste and comparing it to that of using anaerobic digestion of food waste to produce methane.

CHAPTER 2:

MATERIALS AND METHODS

“Though this be madness, there is method in it.”

William Shakespeare

2.1 Materials and methods: Chapter 3

2.1.1 Materials

All chemicals were purchased from Sigma-Aldrich (UK), aside from yeast extract [10-12% total nitrogen, 5-6% amino nitrogen, <0.5% NaCl] (Melford Laboratories, Ipswich, UK) and peptone (Fisher Bioreagents, Loughborough, UK). All reactants were used as received with no additional purification.

2.1.2 Microorganisms and medium

Rhodotorula glutinis 2439 and *Rhodotorula minuta* 62 were purchased from the National Collection of Yeast Cultures (NCYC, Norwich, UK) and were used throughout the investigation. The yeast strains were maintained on yeast peptone dextrose (YPD) agar plates [10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 15 g/L agar] at 4 °C until used and sub-cultured monthly. For the seed culture, 25 ml of YM medium [3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose, pH 6.5] was inoculated with a single yeast colony in a sterile 50 ml *falcon* tube and was incubated at 28 °C and 180 rpm for 25 h. After the specified incubation period, the inoculum contained $\sim 1 \times 10^8$ c.f.u/ml. All media was made using deionised water and sterilised by autoclaving (121 °C, 15 min).

2.1.3 Culture conditions

Experiments were carried out in 250 ml un-baffled conical flasks with a working volume of 100 ml. The cultures were initiated with 10% (v/v) of the 25-h-old seeding culture medium. The original RSM medium was used throughout [1 g/L yeast extract, 0.1 g/L NaCl, 0.4 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g/L CaCl_2], supplemented with the desired quantity of glucose and $(\text{NH}_4)_2\text{SO}_4$, and incubated at the temperature according to the experimental design in a rotary shaker at the 180 rpm for 120 h.

2.1.4 Design of experiments

A response surface methodology (RSM) using a 3^3 full factorial design¹⁵⁵ was performed to develop mathematical correlations between three independent variables and to approach the optimum response region. The range of the variables

tested was: glucose, 10-30 g/L; $(\text{NH}_4)_2\text{SO}_4$, 0.5-1.5 g/L and temperature, 25-35 °C. According to this design, 30 runs were conducted including three replications at the central point for understanding experimental variance. The actual design of the 27 experiments is given in From this, the coefficient of determination (R^2) of the model for Y_1 , Y_2 , Y_3 and Y_4 were 0.979, 0.987, 1 and 0.999, respectively, indicating that the model explains between 97.9 – 100% of the variability in the response. The root mean squared errors (RMSE) of the models for Y_1 , Y_2 , Y_3 and Y_4 were 0.06 g/L, 1.868% d.wt., 0.632% and 0.487%, respectively, with the predicted sum square error (PRESS) RMSE, a measure of the RMSE calculated after the omission of each data point sequentially to provide an indication of model over-fitting, was acceptable with values of 0.069 g/L, 2.548% d.wt., 1.169% and 0.64% respectively (Table 3.2).

The relationship of the variables was determined by analysing the response surface contour plots using the MATLAB model-based calibration, design of experiments software to fit a radial basis function – multiquadratic (qRBF) regression analysis to the experimental data. The adequacy of the model and statistical significance of the regression coefficients were tested using the analysis of variance (ANOVA). MATLAB SIMULINK model was then used to construct a process flow model to predict the outcome of lipid production for different growth conditions.

2.1.5 Analytical methods

2.1.5.1 Biomass harvesting

Cultures were centrifuged (6000 rpm; 10 min; RT) to pellet the microorganisms. The supernatant was removed and a sample kept for analysis if required. The pellet was washed with deionised water to remove any residual sugar and centrifuged as before. The biomass was resuspended in a minimal volume of deionised water before being added drop-wise to liquid nitrogen to snap-freeze the culture. Finally, the samples were freeze-dried under vacuum overnight at -40 °C (Mini lyotrap, LTE Scientific, UK), before the resulting powder was stored in an air-tight container at -20 °C prior to use.

2.1.5.2 Glucose quantification

Residual glucose within the supernatant following the culture of *Rhodotorula sp.* was determined by means of reverse phase HPLC using a Shimadzu 10AVP HPLC system (Shimadzu corp., Japan) equipped with a RID-10A refractive index detector. All samples were filtered through a 0.20 µm filter membrane (Millipore, UK) before analysis. 20 µl of the sample (diluted accordingly) was injected onto an Aminex HPX-87H column (BioRad, CA, USA) and eluted isochratically over a 15 min period with 0.6 ml/min of 0.2 µm-filtered and degassed 5 mM H₂SO₄ at 65 °C. Glucose was quantified using the integration of the peak at 8.9 min and compared to previously developed standard curves within in the range 0.5 – 50 g/L (Appendix A).

2.1.5.3 Lipid extraction

The lipid content of the cells was quantified according to a modified method of Bligh and Dyer.¹⁵⁶ Lipid from 0.1 g of the freeze-dried biomass was extracted using a 2:1 mixture of chloroform : methanol (approximately 20 ml), under reflux at 80 °C for 16 h. 100 µl of concentrated H₂SO₄ was also added to catalyse the transesterification of the lipid into the corresponding fatty acid methyl esters (FAMES). Following the extraction of the lipid, the remaining biomass was separated from the organic fraction using a Whatman filter (Whatman, UK), before the organic solvent was quenched with distilled water to remove the methanol and acid catalyst, and the microbial lipids extracted into the chloroform layer. This was repeated three times to ensure all available lipid was removed before the chloroform was finally removed under vacuum prior to analysis.

2.1.5.4 Lipid quantification

The lipid content and FAME profile were calculated by gas chromatography mass spectrometry (GC-MS) calibrated to known standards. The GC-MS analysis was carried out using an Agilent 7890A Gas Chromatograph equipped with a capillary column (60 m × 0.250 mm internal diameter) coated with DB-23 ([50%-cyanopropyl]-methylpolysiloxane) stationary phase (0.25µm film thickness) and a helium mobile phase (flow rate: 1.2 ml/min) coupled with an Agilent 5975C inert MSD with Triple Axis Detector. A portion of the biodiesel samples (approximately 50 mg) was initially dissolved in 10 ml 1,4-dioxane and 1 µl of this solution was loaded onto the

column, pre-heated to 150 °C. This temperature was held for 5 minutes and then heated to 250 °C at a rate of 4 °C min⁻¹ and then held for 2 minutes. The FAME concentration within the samples was determined using the integration values resulting from the flame ionisation detector and previously calibrated FAME standards (FAME mix C₈ – C₂₄, Supelco, Bellefonte, USA).

2.1.5.5 Biomass and lipid coefficients

Final lipid yield on substrate, $Y_{l/s}$ was calculated according to Equation 2.1 where Δl and ΔS was the change in lipid concentration and substrate, respectively, during the fermentation.

$$Y_{l/s} = -\frac{\Delta l}{\Delta S} \quad (\text{Eqn. 2.1})$$

Final biomass yield on substrate, $Y_{b/s}$ was calculated according to Equation 2.2 where Δb and ΔS was the change in biomass concentration and substrate, respectively, during the fermentation.

$$Y_{b/s} = -\frac{\Delta b}{\Delta S} \quad (\text{Eqn. 2.2})$$

2.1.6 Fuel analysis

2.1.6.1 Viscosity

The kinematic viscosity was measured with Cannon-Fenske routine viscometers No. 75 and 150 (Cannon Instrument company, Pennsylvania, USA), in accordance with standard test methods set out in ASTM D445 and ISO 3104 at 40 °C. Dynamic viscosity was selected over kinematic viscosity when the viscosity of the oil was very high. The dynamic viscosity of the samples were measured using a Bohlin C-VOR torque rebalance rotating disc rheometer (Malvern instruments, Malvern, UK) at 40 °C and the resulting viscosity was converted into kinematic viscosity (Equation 2.3).

$$\text{Kinematic viscosity (mm}^2\text{s}^{-1}) = \frac{\text{Dynamic viscosity (cP)}}{\text{density (g.cm}^{-3})} \quad (\text{Eqn. 2.3})$$

2.1.6.2 Cloud point and density

Cloud points were measured by cooling the samples by 1 °C min⁻¹, holding at each temperature for 10 min and observing any solid formation by eye. Density was

calculated by measuring the mass of 1 ml of the oil on a benchtop scale to an accuracy of 0.5×10^{-4} g.

2.1.6.3 Energy Density

The energy content of the oil and biodiesel were determined using a Parr 1341 plain jacket non-adiabatic bomb calorimeter using a Parr 1108 oxygen combustion bomb (Parr Instrument Company, Illinois, USA). Approximately 0.3 g of each sample was placed in the crucible within the bomb and then the bomb filled with oxygen to a pressure of approximately 25 bar. The oil was ignited using an electronic fuse of standard length and the temperature change of the water within the stirred calorimeter was determined to an accuracy of 0.5×10^{-3} °C.

2.2 Materials and methods: Chapter 4

2.2.1 Materials

All reagents used within Chapter 4 were purchased from Sigma Aldrich (UK), except for D-glucose (Alfa Aesar, Heysham, UK), sodium chloride (VWR, Lutterworth, UK), and the colourimetric ammonium test kits (Randox Laboratories, Crumlin, UK).

2.2.2 Inoculating culture and growth medium

Inoculating cultures of *Rhodotorula glutinis* 2439 and *Rhodotorula minuta* 62 were prepared as described previously (Section 2.1.2). The previously defined RSM medium (Section 2.1.3) was used as the culture medium, supplemented with 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ and 30 g/L glucose.

2.2.3 Bioreactor fermentation conditions

A 2.5 L stirred bioreactor (Bioflow/CelliGen, New Brunswick Scientific, East Brunswick, NJ, USA) was used, with a working volume of 1.5 L. The vessel was constructed with vertical baffles, equally spaced around the periphery of the vessel. The central shaft supported two, 6 bladed disc turbine agitators.

The fermentations were run as aseptic batch cultures. The air inlet port was installed with a sterile hydrophobic membrane air filter (0.2 μm ; Millipore, MA, USA). The assembled bioreactor was filled with 1.5 L of the RSM medium with 30

g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$, and autoclaved (121 °C, 15 min) with the pH and dissolved oxygen probes installed. The pH probe (405-DPS, Mettler Toledo, Switzerland) was calibrated using pH 7.0 and 4.0 at room temperature prior to autoclaving. The concentration of the dissolved oxygen (DO) in the culture medium was measured online using an InPro® 6000 O₂ sensor (Mettler Toledo, Switzerland). The DO probe was calibrated at 28 °C in the sterilised culture medium. For the calibration, the air flow was set to 2.5 vvm, with the impellor rotating at 800 rpm. Once the measured concentration of dissolved oxygen had stabilised, the DO readout was adjusted to an air saturation value of 100%. For the 0% readout, the DO electrode was disconnected from the bioreactor control unit and set at 0% once the system had stabilised.

The sterile bioreactor was inoculated with 50 ml (3.2% by vol) of the previously specified inoculum through the media port. The final volume of the broth in the fermenter after inoculation was 1550 ml. The fermenter was controlled at 28 °C automatically using an electronic heating jacket and cooling recirculating water. Depending on the conditions used the agitation speed was set at: **a.** 800 rpm, with an aeration rate of 2.5 vvm; or **b.** Cascading agitation with a minimum dissolved oxygen content of 25% (maximum and minimum agitation of 700 and 200 rpm, respectively). 1ml of sterile (121 °C, 15 min) polypropylene glycol antifoam was added at the beginning of the fermentation to prevent foam formation. Data was logged every 15 min using Biocommand track and trend software.

Each batch fermentation was run for 140-160 h and 10 ml samples were taken periodically. The optical density was measured immediately after sampling at 590 nm (Colorimetric model 45, Fisher, Loughborough, UK). The samples were centrifuged at 8000 rpm (7600 g) for 10 min (Sorvall™ RC 6 Plus Centrifuge, Thermo Scientific, Loughborough, UK) to pellet the cells. The supernatant was removed and the cell pellet and supernatant stored at -18 °C for further analysis.

2.2.3.1 pH controlled experiments

To understand the effects of pH control on the growth and lipid productivity of *R. glutinis*, for the pH-controlled condition, the pH was maintained at 5.5 ± 0.2

automatically with the addition of 2M potassium hydroxide and 2M phosphoric acid. For the non-pH controlled fermentation, the pH was measured throughout but not controlled. In both fermentations, the starting pH was 5.6 and the dissolved oxygen was measured throughout but not controlled.

2.2.3.2 Sonication experiments

A 2.75 L USC300TH ultrasonic cleaning bath (VWR, Lutterworth, UK) with a frequency of 45 kHz and maximum power input of 80 W was used throughout the study. The sonication cell had a cell volume of 157 ml and was autoclaved (121 °C, 15 min), before being connected to the bioreactor aseptically using sterile silicon tubing (Figure 4.21). The sonication chamber was immersed into the ultrasonic water bath and held approximately 2 cm from the base of the water bath. Several drops of liquid detergent were also added into the water of the ultrasonic bath to decrease the surface tension and thus to provide efficient transfer of the ultrasonic waves. The medium from the bioreactor was recirculated continuously from the point of starting the sonication through the sonication chamber through the use of a peristaltic pump (Watson Marlow 323 bench top pump, Falmouth, UK). The recirculation flow rate was 0.9 L/min (400 rpm) with a residence time in the sonication cell of 10.46 sec.

2.2.4 Analyses

Biomass, glucose concentration and lipid concentration along with the biomass and lipid coefficients were measured and calculated as described in Section 2.1.5.

Specific growth rate, μ , determining the increase in cell mass per unit time was calculated according to Equation 2.4 where X_1 is the biomass at t_1 and X_2 is the biomass concentration at time t_2 during exponential growth.

$$\mu = \frac{1}{(t_1 - t_2)} \ln \frac{X_2}{X_1} \quad (\text{Eqn 2.4})$$

2.3 Materials and methods: Chapter 5

2.3.1 Microbial cultivation

R. glutinis 2439 and *R. minuta* 62 was maintained on YPD agar, with seed cultures cultivated in YM medium as per Section 2.1.1.

2.3.2 Diauxic growth studies

The 26-hour-old seed cultures were centrifuged to pellet the cells (6000 rpm, 10 min) and the supernatant discarded to prevent the transfer of residual sugar. The cell pellet was resuspended in 25 ml RSM medium containing 1 g/L $(\text{NH}_4)_2\text{SO}_4$ but without sugar. 5 ml of this cell suspension was inoculate into 45 ml RSM medium containing 1 g/L $(\text{NH}_4)_2\text{SO}_4$ and 30 g/L of sugar (glucose and xylose) at the ratios given in Table 5.18, in 100 ml Erlenmeyer flasks. The cultures were incubated at 28 °C, 180 rpm for 120 h. 1 ml samples were removed every 24 h and the O.D._{600} measured using a spectrophotometer (Spectronic 200, Thermo Scientific UK). The yeast biomass was removed by centrifugation (14000 rpm, 2 min) and the sugar uptake was assessed using HPLC (detailed in Section 2.1.5.1). All cultures were repeated in triplicate.

2.3.2.1 Kinetic analysis of glucose and xylose assimilation

Based on first-order kinetics, Equation 2.5 was used to analyse the relationship between the reactant rate and the reactant concentration. r_A is the volumetric rate of reaction, k is the first-order rate constant and C_A is the concentration of the reactant; in this case the concentration of glucose or xylose.¹⁵⁷

$$r_A = kC_A \quad (\text{Eqn. 2.5})$$

Given that the system was closed and of a constant volume, the sugar concentration as a function of time in relation to r_A can be expressed as Equation 2.6.

$$r_A = -dC_A/dt \quad (\text{Eqn. 2.6})$$

By integrating Equations 2.5 and 2.6 with initial concentrations of $C_A = C_{A0}$ at $t = 0$, and taking the natural log of both sides, produces Equation 2.7.

$$\ln C_A = \ln C_{A0} - kt \quad (\text{Eqn. 2.7})$$

This was plotted with respect to time in order to determine the rate constant, k , for the reaction.

2.3.3 Alternative sugar studies

The ability of *Rhodotorula* sp. to assimilate a variety of simple sugars commonly present in lignocellulose hydrolysates (glucose, xylose, arabinose, cellobiose, lactose and sucrose) was assessed using a 96-well microtitre plate system. 5 μ l of a 26-hour old seed culture in YM was added to 195 μ l of RSM medium containing 30 g/L glucose and 1 g/L $(\text{NH}_4)_2\text{SO}_4$ in the 96-well plates. After 120 h at 28°C, 180 rpm, the increase in O.D.600 was measured using a plate reader (Versamax, Molecular devices UK). The sugars were tested individually, and in a 50:50 (w/w) combination with glucose and xylose. Each combination was tested using six repeats.

2.3.4 Microbial inhibitor studies

The growth of *Rhodotorula* sp. when single inhibitor compounds were present (furfural, 5-HMF, acetic acid, formic acid, levulinic acid) were examined under aseptic conditions using a 96-well microtitre plate system. 200 μ l of culture were made up in 96-well plates in RSM medium containing 30 g/L glucose, 1 g/L $(\text{NH}_4)_2\text{SO}_4$ and either a low, medium or high level of inhibitor (Table 5.20) sterilised by UV for 1 hour prior to inoculation. The cultures were incubated at 28 °C, 180 rpm the increase in the O.D._{600nm} was measured after 7 days using a plate reader (Versamax, Molecular devices UK).

2.3.5 *Miscanthus* hydrolysate

The *Miscanthus giganteus* grass was depolymerised using five separate methods. This was kindly provided by Hussein *et al.* in the Department of Biology and Biochemistry, University of Bath. Method one (hydrolysate #1); 5% (w/v) of *Miscanthus* was autoclaved for 2 hours in 0.8% sodium sulphate solution (Na_2SO_4), and the pH was then adjusted to 5.5. The insoluble biomass was dried at 100 °C for

1.5 days, and then hydrolysed with the enzyme CTEC-2, in 5 mM of Na_2OOCH_3 buffer at pH 5, for 3 days. Hydrolysate #2 was prepared using a 5% (w/v) of *Miscanthus*, which was autoclaved for 2 hours in 0.8% sodium sulphate solution (Na_2SO_4), and the pH was then adjusted to 5.5. The soluble fraction was then hydrolysed with the enzyme CTEC-2 for 3 days. Hydrolysate #3 was prepared by soaking *Miscanthus* in ammonium hydroxide for 24 hours prior to the depolymerisation. After the 24 hours the *Miscanthus* was autoclaved (5% (w/v)) for 2 hours in 0.8% sodium sulphate solution (Na_2SO_4), and the pH adjusted to 5.5. The insoluble biomass was dried at 100 °C for 1.5 days, and then hydrolysed with the enzyme CTEC-2, in 5 mM of Na_2OOCH_3 buffer at pH 5, for 3 days. Hydrolysate #4 was prepared by soaking *Miscanthus* in ammonium hydroxide for 24 hours prior to the depolymerisation. After the 24 hours the *Miscanthus* was autoclaved (5% (w/v)) for 2 hours in 0.8% sodium sulphate solution (Na_2SO_4), and the pH adjusted to 5.5. The soluble fraction was then hydrolysed with the enzyme CTEC-2 for 3 days. Hydrolysate #5 was prepared by soaking *Miscanthus* in ammonium hydroxide for 24 hours, then treating with the enzyme CTEC-2 in 5 mM of Na_2OOCH_3 buffer at pH 5, for 3 days. The hydrolysates 1-5 were then autoclaved (121 °C, 15 min.) to ensure sterility prior to inoculation.

2.3.5.1 Cultures on depolymerised *Miscanthus*

A 26-hour-old seed culture of *R. minuta* grown in YM medium (28 °C, 180 rpm) was used to inoculate 20 ml of each of the five hydrolysates (10% (v/v)). The cultures were grown at 28 °C, 180 rpm for 168 h in duplicate. 0.5 ml samples were taken every 24 h to measure the O.D.₆₀₀. Residual sugar concentrations were measured by HPLC analysis (Section 2.1.5).

2.3.6 Preparation of food waste

Using the data available from Wrap,¹⁵⁸ a standard food waste was developed (drink waste excluded), with consumables purchased from a Sainsbury's supermarket consisting of: 263 g of boiled Sainsbury's carrot, broccoli and cauliflower mix, 357 g of boiled baking potato (skin on), 95 g apple, 65 g orange peel, 150 g banana, 100 g slices white bread, 100 g Sainsbury's basics sliced cooked chicken, 50 g Sainsbury's basics crunchy salad, 160 g used tea bags and 90 g Sainsbury's basics ready-

prepared chilled lasagne. These were macerated using a conventional food processor, until a homogenised substrate was obtained. This mix was blended 1:1 (w/w) with deionised water using a household blender. This mix was then subjected to different hydrolysis techniques (see below).

2.3.6.1 Enzymatic hydrolysis

The food waste mixture was adjusted to the desired pH according to the experimental design using sodium hydroxide and hydrochloric acid. The blends were stored at 4 °C until use, but for no longer than one week. Celluclast® 1.5L cellulase mix (Novozymes, Bagsværd, Denmark), derived from *Trichoderma reesei* cellulase is widely utilised for the hydrolysis of lignocellulostic biomass feedstocks,¹⁵⁹ and thus was used to hydrolyse the carbohydrate present in the food waste. The enzyme mixture, consisting predominantly of cellobiohydrolases (CBHs) and endo-1,4- β -glucanases (EGs) catalyses the breakdown of cellulose into glucose, cellobiose and other glucose polymers. Originally isolated from decaying canvas during World War II, *T. reesei* is now one of the most well-known and researched cellulose-producing fungi.¹⁶⁰ The optimum conditions for activity of this enzyme preparation are in the range pH 4.5 – 6.0 and 50 – 60 °C.

Enzymatic hydrolysis experiments were conducted in 100 ml Erlenmeyer flasks each containing 50 ml minced food waste, in a shaking incubator at 200 rpm. Enzyme loading, pH, incubation temperature and time were done according to the experimental design. Following incubation, the food waste mixture was centrifuged (4000 rpm, 20 min) to remove the solid biomass and the supernatant removed. Enzymatic activity was stopped by autoclaving the supernatant (121 °C, 15 min). The resulting solids were removed by centrifugation (4000 rpm, 20 min) before the supernatant was filtered through Whatman No.1 filter paper (Whatman, Maidstone, UK). This final translucent hydrolysate was autoclaved again (121 °C, 15 min) to ensure sterility of the culture medium.

2.3.6.2 Thermochemical hydrolysis

Acid hydrolysis was performed according to a modified method of Chi *et al.*,⁸⁴ in which 3% (v/v) concentrated sulphuric acid was added to the blended food mixture

and autoclaved (121 °C, 15 min) to hydrolyse the polysaccharides within the food waste. The autoclaved mixture was filtered twice through a Whatman No. 1 filter paper (Whatman, Maidstone, UK) before being neutralised with sodium hydroxide to pH 6.5. The resulting precipitate was again filtered twice through Whatman filter paper to yield a translucent food waste hydrolysate (FWH), which was autoclaved (121 °C, 15 min) to ensure sterility.

2.3.7 Analyses

Biomass, glucose concentration and lipid concentration, alongside the lipid and biomass coefficients were analysed according to Section 2.1.5.

2.4 Materials and methods: Chapter 6

2.4.1 Materials

All materials were purchased from Sigma-Aldrich (UK) aside from the deuterated chloroform (Flurochem, Hadfield, UK).

2.4.2 Microbial cultivation

R. glutinis 2439 was cultured aerobically in a 1.5 L jacketed airlift fermenter, with a working volume of 1 L at 30 °C. The previously defined YM medium was used throughout (Section 2.1.1), and the culture was initiated with 10% (v/v) of an overnight seeding culture. After 168 h of culture, the yeast biomass was concentrated through settling, the supernatant removed and replaced with a sterile 2% (w/v) glucose solution to promote lipid accumulation. This second stage of the fermentation was maintained for 120 h. On completion, the yeast biomass was removed by centrifugation (6000 rpm, 10 min), the supernatant removed, and the biomass washed with deionised water to remove any residual sugar. The biomass was pelleted into liquid nitrogen before being freeze dried (-40 °C) overnight. The resulting powder (9.1 g) was stored in an air-tight vial at -20 °C prior to use.

2.4.3 Oil Extraction

2.4.3.1 Soxhlet extraction

0.1 g of the freeze-dried microbial biomass was placed inside a cellulose thimble (Whatman, Maidstone, UK) within the Soxhlet apparatus. Using an adapted method

from Bligh & Dyer,¹⁵⁶ a 2:1 mixture of chloroform and methanol (total 50 ml) was set to reflux at 85 °C for the desired amount of time (0.5, 1, 2, 4, 8, 12, 24 or 48 h). The volatile solvent was removed under vacuum, before 20 ml methanol and 0.1 g conc. sulphuric acid (catalyst) was added to the resulting oil and set to reflux at 85 °C for 8 h. The excess methanol was removed under vacuum and the lipid extracted into chloroform. The organic layer was washed with water to remove the acid catalyst, glycerol and methanol, before the volatiles were removed under reduced pressure prior to analysis.

2.4.3.2 Microwave extraction

Microwave extraction was performed using an Anton Paar monowave 300 microwave reactor equipped with a MAS 24 autosampler (Anton Paar Ltd., St Albans, UK). 0.1 g of the microbial biomass was extracted into a 2:1 mixture of chloroform:methanol (6 ml total) within a 10 ml sealed, stirred microwave vial, capable of withstanding a pressure of 30 bar. Concentrated sulphuric acid was used as the catalyst at 1 wt.% (0.001 g), 10 wt.% (0.01 g), 25 wt.% (0.025 g) or 100 wt.% (0.1 g). The microwave was set on an automated cycle comprising three steps: 1. Heating to the desired temperature and pressure (typically lasting < 1 min) with 1000 rpm stirring; 2. The reaction (0.5 – 20 min, 1000 rpm stirring); 3. Fast cooling using compressed N₂ (typically < 2 min.). The biomass was separated with filter paper (Whatman, UK) and the organic mixture quenched with water. The chloroform was washed three times with water before the chloroform was removed under vacuum prior to analysis.

2.4.4 ¹H NMR spectroscopy

The FAME conversion was calculated by dissolving a fraction of the sample in CDCl₃ and analysing by ¹H NMR spectroscopy in an adapted method by Knothe.¹⁶¹ This method uses the integration of the peaks assignable to the reactants and products. The peak assignable to the methoxy group of the FAME (δ 3.6 ppm) was compared to the glyceride backbone (δ 4.0 – 4.5 ppm) of the triacylglyceride (Equation 2.8).

$$FAME\ conversion\ (\%) = \left[\frac{5a}{5a+9b} \right] \times 100 \quad (\text{Eqn. 2.8})$$

Where a is the integration of the methoxy peak at δ 3.6 ppm, and b is the integration of the glyceride peak at δ 4.0 - 4.5 ppm. This method is capable of quantification of products with an accuracy of $\pm 2\%$.¹⁶² NMR spectroscopic measurements were carried out at 298 K using a Bruker AV300 spectrometer, operating at 300 MHz. The lipid content and FAME profile were calculated by GC-MS, as described in Section 2.1.5.4.

2.4.5 Energy return on investment

The power used for the microwave was calculated by integrating the energy output from the microwave using the trapezium rule (Eqn. 2.9) with a step change of 0.05.

$$Area = \frac{h}{2} [y_0 + 2(y_1 + y_2 \cdots + y_{n-1}) + y_n] \quad (\text{Eqn. 2.9})$$

Where: h is 0.05 (step size), y_0 is the power at time 0, y_1 is the power at 0.05 sec. y_2 is the power at 0.1 sec., etc. and n is the number of steps.

The experimental data from the microwave extractions was used to calculate the EROI based on both lipid extraction and the biodiesel produced.

$$Energy\ from\ oil\ (MJ) = Mass\ of\ oil\ (kg) \times Energy\ density\ (MJ\ kg^{-1}) \quad (\text{Eqn. 2.10})$$

$$Mass\ of\ oil\ (kg) = Mass\ of\ yeast\ (kg) \times Lipid\ recovered\ (\%) \quad (\text{Eqn. 2.11})$$

In which the energy density of the oil was 39.99 MJ/kg and the mass of yeast was maintained at 1×10^{-4} kg. The EROI was then determined using the relationship between the usable energy in the oil compared to the energy expended during the extraction process (Equation 2.12).

$$EROI\ (\%) = \left[\frac{Energy\ from\ the\ oil}{Energy\ input\ into\ microwave} \right] \times 100 \quad (\text{Eqn. 2.12})$$

Calculations for an increased amount of lipid were undertaken assuming the same power output, irrespective of the level of lipid extracted.

CHAPTER 3:

**TAILORING THE LIPID PROFILE OF *RHODOTORULA* SPECIES
THROUGH OPTIMISATION OF THE ENVIRONMENTAL
CONDITIONS**

“The way to get started is to quit talking and begin doing.”

Walt Disney

3. Preamble

This chapter describes the growth of *Rhodotorula* species on refined glucose. This also includes an understanding of how the environmental growth conditions (temperature and C/N ratio) can affect the resulting oil and how this can be tailored to suit the desired application. The results from this chapter have been presented as a publication which can be found in the appendix:

L. A. Sargeant, C. J. Chuck, J. Donnelly, C. D. Bannister, R. J. Scott, **2014**, Optimizing the lipid profile, to produce either a palm oil or biodiesel substitute, by manipulation of the culture conditions for *Rhodotorula glutinis*. *Biofuels*, 5 (**1**), 33-34.

3.1 Introduction

The majority of research into microbial lipids has focussed on improving the economic competitiveness of the lipids compared to plant- and animal-derived oils.²⁰ As the *molecular toolkit* within biochemistry and molecular biology has developed, the potential to genetically modify microorganisms has received ever-growing attention to improve lipid yields or to generate oils with tailored fatty acid profiles.¹⁶³ However, public concern about the use of genetically modified organisms (GMOs) and the stability of engineered strains within industrial microbial processes are important considerations. The cost surrounding the development of these GMOs is also a significant factor. Therefore, another promising approach to deliver tailored lipids for application is to direct the production of suitable lipids through the manipulation of the growth conditions.

3.1.2 Improvement of microbial lipid production

For a given yeast strain, the total lipid yield and composition of the accumulated fatty acids are strongly influenced by growth parameters including the carbon source, C/N molar ratio, other nutrients, oxygen level, pH and temperature.¹⁶⁴ One of the most important factors for lipid accumulation in oleaginous microorganisms is the change in the intracellular concentration of various metabolites, due to the exhaustion of some nutrients in the culture medium.¹⁷ For non-oleaginous yeast,

nitrogen limitation hinders cell growth and the excess carbon is channelled into polysaccharide synthesis, such as starch and β -glucan.¹⁶⁵ In oleaginous yeast, nitrogen exhaustion initiates a series of metabolic steps leading to *de novo* lipid biosynthesis in which the carbon source is used for lipid accumulation rather than cell proliferation processes.^{22,47,164-165} Lipid accumulation is influenced by the C/N ratio, with lipid accumulation induced at molar ratio C/N > 20,⁸⁶ but with an optimum being close to 100.³⁸ Indeed, the lipid yield from *R. glutinis* more than doubled when the C/N ratio was increased from 20 to 70, but a further increase to C/N 120 did not lead to higher lipid yields.⁷⁶ One exception is the yeast, *Cryptococcus terricola* which has been shown to accumulate intracellular lipids during the logarithmic growth phase under nitrogen-rich conditions.⁵¹ Nitrogen limitation is not the sole factor which can trigger the accumulation of lipids in oleaginous microorganisms. For example, lipid accumulation in *Rhodospiridium toruloides* was induced in nitrogen-rich medium when sulfate or phosphate became the limiting factor of cell growth.^{87,166}

3.1.3 The need for tailored lipids

The most common fatty acids contained within yeast and plant oils are palmitic acid (16:0), steric acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). The relative proportion of these lipids does, however, depend on the feedstock used. For example, palm oil contains approximately 55% saturated lipids and thus the resulting oil offers a high viscosity and melting point which is ideal for applications within the healthcare and food industries.¹⁶⁷ It is also ideal for upgrading into surfactants. For the automotive industry, saturate-rich esters enhance oxidative stability and the cetane number of the fuel (providing better ignition quality) but they also possess poor cold-flow properties.¹¹⁷ For the aviation industry, the high level of saturation within this type of feedstock would require less hydrogen to fully saturate the oil in order to produce a HEFA fuel (hydroprocessed esters and fatty acids). This would in turn reduce the processing cost. Conversely, an oil rich in polyunsaturated fatty acids, such as that from linseed oil, which is rich in linolenic acid (18:3) would offer a lower melting point, thus enhancing the low-temperature properties if the oil were transesterified into

biodiesel. However, this biodiesel would also have reduced oxidative stability and a poor cetane number, and thus the 18:3 fatty acid component within biodiesel is limited to 12 mol.% according to EN 14-214 specification. Similarly, fatty acids with four or more double bonds are limited to 1 mol.% for the same reasons. Oils rich in polyunsaturates are however highly desirable in the polymer industries as the double bonds are easily cross-linked. Linolenic acid is also used within the homeopathic community as a dietary supplement due to alleged health benefits linked to its consumption.

One of the most desirable fatty acids for the transport industry is oleic acid (18:1), which is found in concentrations of approximately 60% in rapeseed oil,¹¹⁷ and as such it is the main feedstock for biodiesel production in the EU and China.¹⁶⁸ Methyl oleate has a melting point and kinematic viscosity (at 40 °C) of -20 °C and 4.51 mm²/s, respectively,¹¹⁷ which sits comfortably within EN 14-214 biodiesel specification. Methyl oleate also offers a high energy density (40.01 MJ/kg), superior ignition quality as well as improved oxidative stability.¹¹⁶ Oleic acid is also of interest for the production of platform chemicals and potential fuel molecules as the cross metathesis of methyl oleate with ethene yields methyl 9-decanoate and 1-decene.¹⁶⁹ 1-decene has been recently explored as a drop-in fuel replacement for the aviation industry as it is one of the few uni-molecular fuels obtainable from biomass that meets the fuel properties outlined for Jet A-1 (DEFSTAN 91-91). A further benefit is that 1-decene offers an energy density 10% higher than that of Jet A-1.¹⁷⁰ Methyl decanoate offers the potential as a suitable blend agent within biodiesel, and it can also be used in the manufacture of surfactants, lubricants and polymers.¹⁶⁹

3.1.3.1 Tailored lipids from yeast

Due to the inherent physical differences resulting from the varying oil feedstocks, it is imperative that the replacement yeast oil has a fatty acid profile similar to the oil that it is displacing. The fatty acid profile of oleaginous yeast can vary between species, among strains of the same species, as well as for the same strain grown under different environmental conditions.²¹ A number of techniques have been used previously to tailor the lipid profile of oleaginous yeast. In order to produce a

saturate-rich oil for use as a cocoa butter or palm oil replacement, crystallising-out the saturated fats at low temperatures, including $\Delta 9$ and $\Delta 12$ desaturase inhibitors in the culture medium,¹⁵⁰ genetic manipulation,¹⁷¹⁻¹⁷² and O₂ depletion¹⁷³ have been used. One of the simplest methods to tailor the lipid profile is through the manipulation of the growth conditions to yield the desired lipid profile. For example, a higher C/S molar ratio favoured the production of saturated fatty acids in *R. toruloides*.⁸⁷ Temperature-induced variations in the fatty acid profile of the yeast *C. oleophila*, *C. utilis* and *R. toruloides* have been demonstrated, but the effects were species specific.¹⁷⁴ Reducing the culture temperature for *C. curvatus* increased the amount of saturated esters by 10%,¹⁷⁵ while the amount of polyunsaturates in various yeast of the *Zygomycete* genera were reduced substantially at lower growth temperatures.¹⁷⁶ In contrast, lower incubation temperatures were reported to increase the level of polyunsaturates in *R. glutinis*¹⁷⁷ and *Y. lipolytica*.¹⁷⁸

3.1.4 Design of experiments

One method to optimise the production of microbial lipids is through processes such as Design of Experiments (DoE). Statistical experimental design techniques, especially the response surface methodology (RSM) are extremely useful in understanding the effects and interaction of multiple factors. RSM has also been used widely for biotechnological and industrial process optimisation.^{72,179} Medium optimisation of lipid production using RSM has been used previously for oleaginous microorganisms including *R. glutinis* grown on crude glycerol,⁷² and the co-fermentation of glucose and xylose using *L. starkeyi*.¹⁷⁹ Similarly, temperature can affect the lipid profile in various oleaginous microorganisms,¹⁷⁴ though this has not been used to assess the lipid profile of *Rhodotorula* species.

3.1.5 Aim of the chapter

Using a response surface methodology, the temperature and C/N ratio of the growth medium were varied to evaluate the effects on the production of either saturated or monounsaturated esters, and whether this could be used to tailor the lipid profile of *Rhodotorula* species for industrial application.

3.2 Results and discussion

3.2.1 Tailoring the lipid profile of *R. glutinis*

A response surface methodology using a 3^3 full factorial design was performed to develop mathematical correlations between three independent variables and to approach the optimum response region.¹⁵⁵ The range of the variables tested were: glucose, 10 - 30 g/L; $(\text{NH}_4)_2\text{SO}_4$, 0.5 - 1.5 g/L and temperature, 25 - 35 °C. The low, medium and high values for the independent variables were coded as -1, 0 and 1, respectively. According to this design, a total of 30 experiments were conducted with three replicates at the central point for assessing experimental variance (Table 3.1). From this, the coefficient of determination (R^2) of the model for Y_1 , Y_2 , Y_3 and Y_4 were 0.979, 0.987, 1 and 0.999, respectively, indicating that the model explains between 97.9 – 100% of the variability in the response. The root mean squared errors (RMSE) of the models for Y_1 , Y_2 , Y_3 and Y_4 were 0.06 g/L, 1.868% d.wt., 0.632% and 0.487%, respectively, with the predicted sum square error (PRESS) RMSE, a measure of the RMSE calculated after the omission of each data point sequentially to provide an indication of model over-fitting, was acceptable with values of 0.069 g/L, 2.548% d.wt., 1.169% and 0.64% respectively (Table 3.2). The correlation of the effects of the input variables on the production of biomass (Y_1), total lipid (Y_2), and production of the fatty acids 16:0 (Y_3) and 18:1 (Y_4) were assessed. A regression analysis was carried out to fit the response function and predict the outcomes using a radial basis function-multiquadratic (qRBF) response surface. The results obtained by the qRBF were analysed by means of the analysis of variance using the 30 experimental data points (Table 3.2). 16 – 19 centres were used to assess the model fit for each of the dependent variables, with the predicted output compared to the measured response (Figure 3.1).

From this, the coefficient of determination (R^2) of the model for Y_1 , Y_2 , Y_3 and Y_4 were 0.979, 0.987, 1 and 0.999, respectively, indicating that the model explains between 97.9 – 100% of the variability in the response. The root mean squared errors (RMSE) of the models for Y_1 , Y_2 , Y_3 and Y_4 were 0.06 g/L, 1.868% d.wt., 0.632% and 0.487%, respectively, with the predicted sum square error (PRESS) RMSE, a measure of the RMSE calculated after the omission of each data point

sequentially to provide an indication of model over-fitting, was acceptable with values of 0.069 g/L, 2.548% d.wt., 1.169% and 0.64% respectively (Table 3.2).

Table 3.1: Experimental range for the three independent variables used in response surface methodology in terms of coded and actual factors, and experimental data for the response surface analysis for *Rhodotorula glutinis*.

Test	Independent Variables			Dependent Variables			
	Temp. (°C)	Glucose (g/L)	(NH ₄) ₂ SO ₄ (g/L)	Biomass (g/L)	Total lipid (dwt%)	% 18:1	% 16:0
	x_1	x_2	x_3	Y_1	Y_2	Y_3	Y_4
1	-1(25)	-1(10)	-1(0.5)	4.518	7.68	55.66	19.67
2	0(30)	-1(10)	-1(0.5)	3.499	5.73	62.45	19.34
3	1(35)	-1(10)	-1(0.5)	1.972	11.47	64.21	17.20
4	-1(25)	0(20)	-1(0.5)	6.708	21.08	47.62	30.09
5	0(30)	0(20)	-1(0.5)	7.177	21.44	59.15	25.23
6	1(35)	0(20)	-1(0.5)	2.421	10.17	67.07	17.05
7	-1(25)	1(30)	-1(0.5)	8.174	29.24	46.46	31.42
8	0(30)	1(30)	-1(0.5)	8.299	31.76	54.53	26.91
9	1(35)	1(30)	-1(0.5)	2.046	11.49	64.54	17.29
10	-1(25)	-1(10)	0(1.0)	3.92	5.99	55.06	18.17
11	0(30)	-1(10)	0(1.0)	3.53	10.02	66.12	18.68
12	1(35)	-1(10)	0(1.0)	1.792	10.74	63.75	17.16
13	-1(25)	0(20)	0(1.0)	6.263	7.55	56.34	25.18
14	0(30)	0(20)	0(1.0)	5.968	19.80	64.43	24.74
15	1(35)	0(20)	0(1.0)	1.852	10.56	64.12	16.81
16	-1(25)	1(30)	0(1.0)	9.022	20.42	50.59	29.84
17	0(30)	1(30)	0(1.0)	7.148	19.15	61.79	25.06
18	1(35)	1(30)	0(1.0)	2.118	10.66	64.36	16.65
19	-1(25)	-1(10)	1(1.5)	3.925	5.43	56.37	18.11
20	0(30)	-1(10)	1(1.5)	3.015	4.44	62.60	19.06
21	1(35)	-1(10)	1(1.5)	2.157	13.10	66.00	16.91
22	-1(25)	0(20)	1(1.5)	6.815	14.22	58.30	24.52
23	0(30)	0(20)	1(1.5)	7.3	14.54	64.90	23.92
24	1(35)	0(20)	1(1.5)	1.984	10.61	65.34	16.38
25	-1(25)	1(30)	1(1.5)	7.957	11.20	56.27	26.39
26	0(30)	1(30)	1(1.5)	ND	21.16	62.56	24.86
27	1(35)	1(30)	1(1.5)	2.392	10.13	66.45	17.00
28	0(30)	0(20)	0(1.0)	5.687	20.68	64.18	24.28
29	0(30)	0(20)	0(1.0)	ND	16.10	64.46	24.14
30	0(30)	0(20)	0(1.0)	7.606	15.48	64.16	24.84

For independent variables, the value given is that used in the model and values in parentheses are the true values. Dwt: Dried weight; ND: No data; x: independent variables; Y: dependent variables.

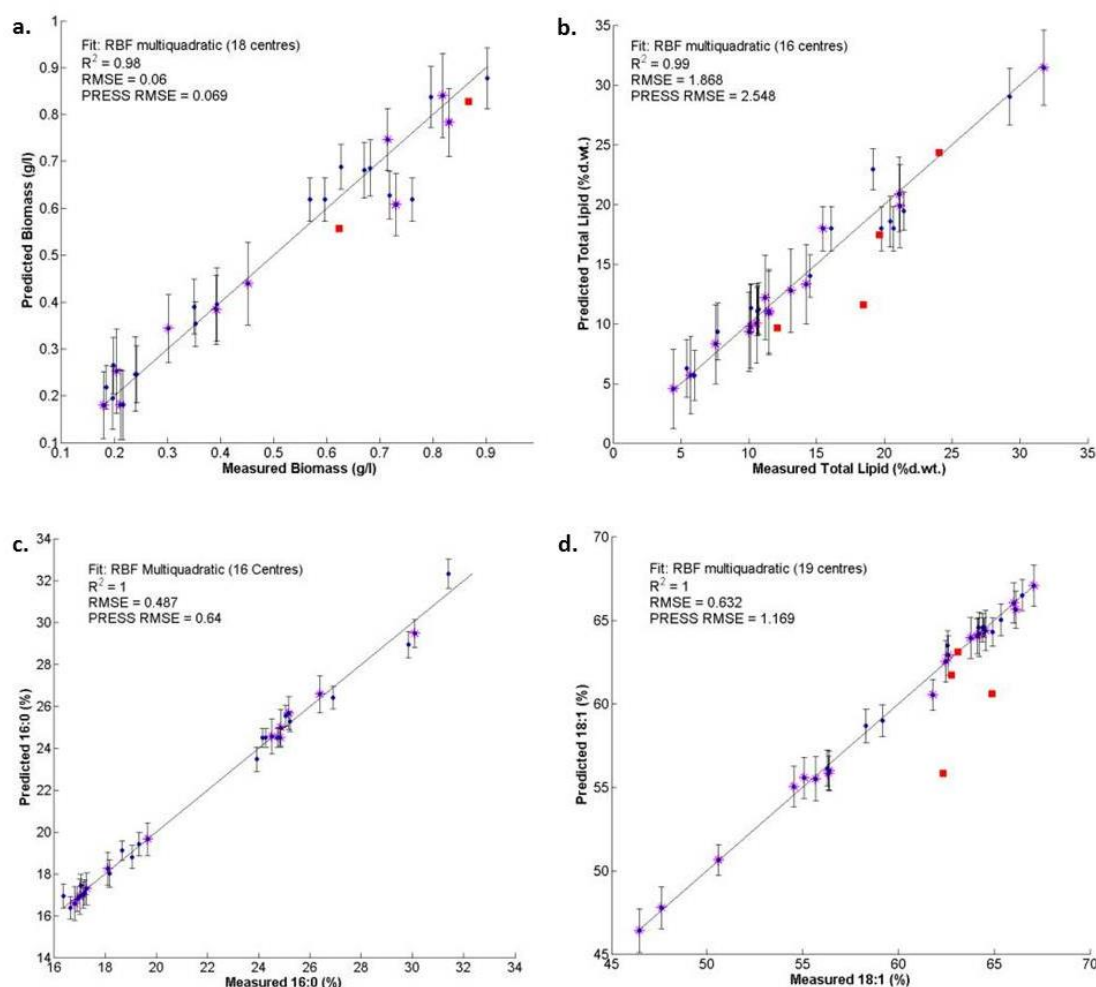


Figure 3.1: Response surface model fits for the main fatty acids, lipid content and total biomass from *Rhodotorula glutinis*. **a.** biomass; **b.** total lipid; **c.** Palmitic acid, 16:0; **d.** Oleic acid, 18:1. The predicted values represent the values obtained from the regression analysis using a radial basis function multi-quadratic response surface. The centres used in the construction of these models are indicated with a magenta star around the data point. Error bars represent the spread of data observed for repeated central point. The red squares indicate validation data not included in the construction of the models. Dwt: Dried weight; PRESS: Predicted sum square error; R^2 : Coefficient of determination; RBF: Radial basis function; RMSE: Root mean square error.

The coefficient of variation (CV) value indicates the degree of precision with which the experiments are compared as it expresses the variation as a percentage of the mean. A high CV value ($> 15\%$) usually lowers the reliability of the experiment. In these models, CV values of 12.42%, 13.28%, 1.04% and 2.63% were observed for biomass, total lipid, percentage 18:1 and percentage 16:0, respectively (Table 3.2). While the CV values for biomass and lipid may be comparatively high, these variables are most affected by the changing environmental conditions and thus results in the largest variation. Low biomass and lipid yields adversely affect the CV

value obtained. For this reason, the CV values indicate reliability in the results. The probability value (P -value) was also calculated to ensure that the results were not due to chance occurrence. When the P -value is less than 0.05 (or more stringently 0.01), the model is considered to be significant. The P -values of the models were all <0.01 indicating a high significance of the coefficients.

Table 3.2: Analysis of Variance (ANOVA) for the independent variables of the response surface-guided experiments for *Rhodotorula glutinis*.

	Source	SS	DF	MS	R^2	RMSE	CV (%)
Y_1 Biomass	Model	7.988	8.648	0.925	0.979	0.06	12.42
	Error	0.07	19.352	3.62E-03			
	Total	8.171	28	0			
Y_2 Total lipid	Model	7184.437	14.593	492.515	0.987	1.868	13.28
	Error	53.778	15.407	3.491			
	Total	7285.084	30	0			
Y_3 Total 18:1	Model	1.11E+05	17.869	6229.903	1	0.632	1.04
	Error	4.85	12.131	0.4			
	Total	1.11E+05	30	0			
Y_4 Total 16:0	Model	14998.972	9.851	1522.544	0.999	0.577	2.63
	Error	6.712	20.149	0.333			
	Total	15008.715	30	0			

SS, Sum of squares; DF, Degree of Freedom; MS, Mean square; RMSE, Root mean-square error; CV, Coefficient of variance.

The regression models were employed to develop three-dimensional response surfaces in order to visualise the combined effects on the dependant variables Y_1 – Y_4 , (Figures 3.2 – 3.5). When the effect of two factors was plotted, the other factor was set at zero (From this, the coefficient of determination (R^2) of the model for Y_1 , Y_2 , Y_3 and Y_4 were 0.979, 0.987, 1 and 0.999, respectively, indicating that the model explains between 97.9 – 100% of the variability in the response. The root mean squared errors (RMSE) of the models for Y_1 , Y_2 , Y_3 and Y_4 were 0.06 g/L, 1.868% d.wt., 0.632% and 0.487%, respectively, with the predicted sum square error (PRESS) RMSE, a measure of the RMSE calculated after the omission of each data point sequentially to provide an indication of model over-fitting, was acceptable with values of 0.069 g/L, 2.548% d.wt., 1.169% and 0.64% respectively (Table 3.2).

3.2.1.1 Biomass

The relationship between glucose and biomass production was relatively simple, with greater quantities of biomass produced at higher glucose concentrations

(Figure 3.2, a & c). The maximum biomass yield was 9.022 g/L (25 °C, 30 g/L glucose, 1.0 g/L (NH₄)₂SO₄), which decreased to 1.792 g/L (35 °C, 10 g/L glucose, 1.0 g/L (NH₄)₂SO₄). Interestingly, the effect of nitrogen concentration on the amount of biomass produced was minimal, with little effect observed compared with temperature and only a slight increase in biomass being produced at low nitrogen concentrations compared with the effect of glucose (Figure 3.2). The effect of temperature on biomass production was considerable, with greater levels of biomass produced at lower temperatures (25 – 30 °C). Irrespective of the other environmental conditions, growth at 35 °C was poor, with a maximum biomass yield at this temperature of 2.421 g/L (35 °C, 20 g/L glucose, 0.5 g/L (NH₄)₂SO₄). While *Rhodotorula sp.* have been shown to grow at temperatures ranging from 20 – 32 °C, for most strains the optimal biomass is produced at 28 °C.^{77,180} This corresponds with this study in which the optimum temperature for biomass production was found to be between 25 and 30 °C.

3.2.1.2 Total Lipid

The total lipid, as measured as a function of dry weight, was greatest at high glucose concentrations and low nitrogen concentrations (Figure 3.3). It has previously been shown that the higher the carbon-to-nitrogen ratio (C/N), the greater the lipid accumulation.⁵⁶ This is due to the up regulation of the enzyme ATP:citrate lyase, an enzyme complex only present in oleaginous microorganisms, which upon nitrogen limitation, increases the metabolic flux of the carbon source into fatty acid biosynthesis.¹⁶⁵ The relationship between the lipid production and temperature, however, is more complex. Irrespective of the C/N ratio, little lipid was produced at high temperatures. The optimal temperature for lipid production was around 30 °C, though similar levels (~20% d.wt) can be obtained at lower temperatures providing the C/N ratio is sufficiently high (generally, C/N >20).⁸⁶ The very low biomass yields observed at 35 °C shows the importance of controlling temperature for lipid production.

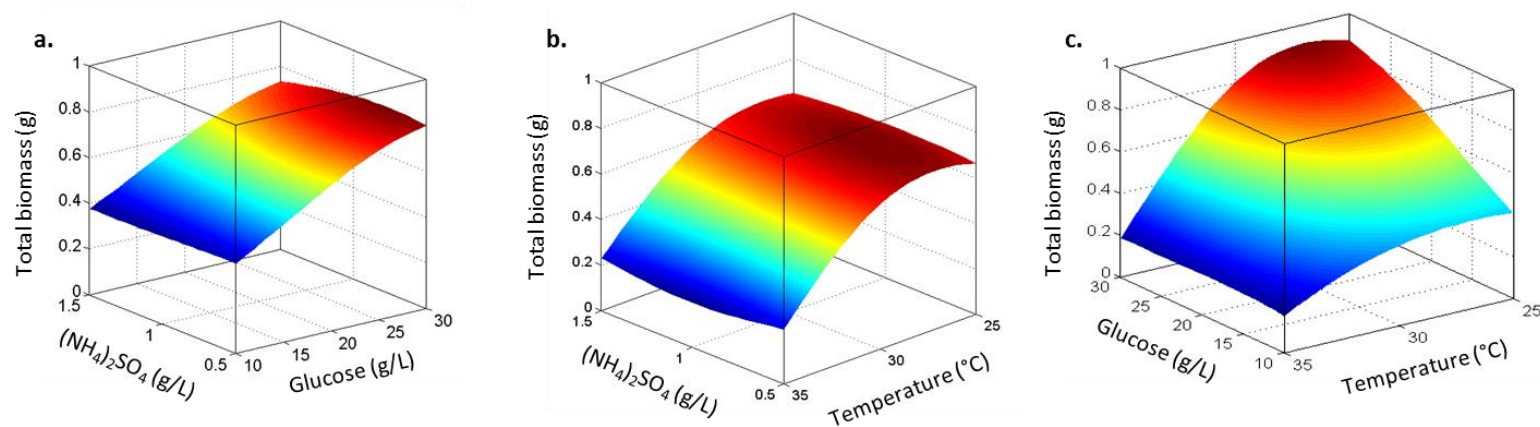


Figure 3.2: Response surface plots for total biomass produced by *Rhodotorula glutinis*. a. Interaction between glucose and $(\text{NH}_4)_2\text{SO}_4$; b. $(\text{NH}_4)_2\text{SO}_4$ and temperature; c. glucose and temperature.

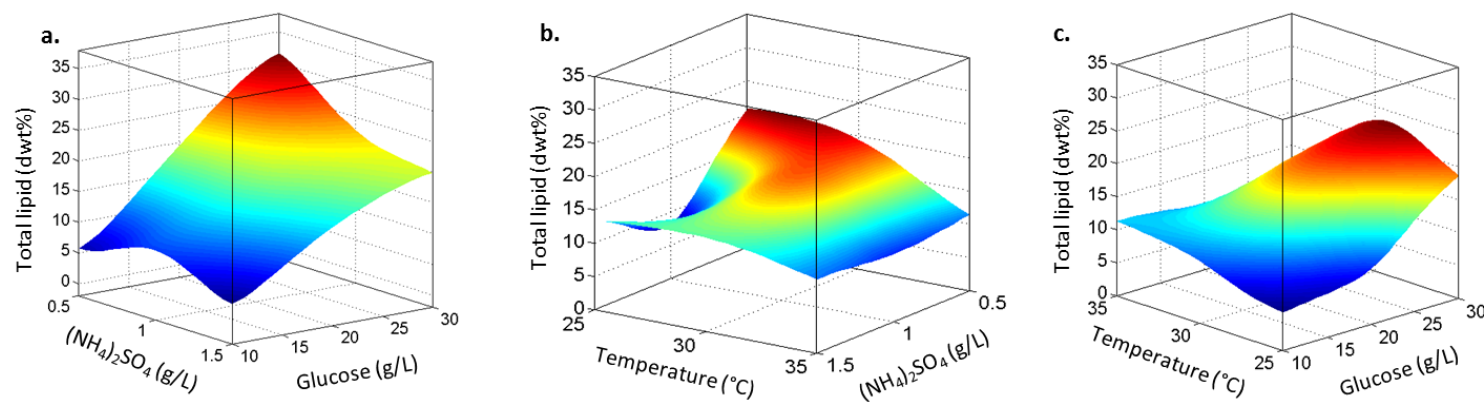


Figure 3.3: Response surface plots for total lipid produced by *Rhodotorula glutinis*. a. Interaction between glucose and $(\text{NH}_4)_2\text{SO}_4$; b. $(\text{NH}_4)_2\text{SO}_4$ and temperature; c. glucose and temperature.

3.2.1.3 Oleic acid (18:1) production

In order to produce a feedstock that is suitable for biodiesel production, the highest possible 18:1 content is required. The predominant biodiesel source within the EU and China is rapeseed oil, which contains between 55 - 65% 18:1.^{117,168} Temperature induced variations in the proportions of oleic acid have been previously observed in *Candida oleophila*, *Candida utilis* and *R. toruloides*, but the effect of the environmental conditions on lipid production were found to be species-specific.¹⁷⁴ In *R. glutinis*, 18:1 production appeared to be favoured by high temperatures (Figure 3.4), with greater than 65% of the lipid being 18:1 at 30 °C or above, irrespective of the level of glucose or nitrogen provided in the culture medium. At lower temperatures (e.g. 25 °C) this fell to as low as 45% depending on the other variables. These findings are in contrast to Ferrante *et al.* and Granger *et al.*, who reported an increase in polyunsaturated fatty acids at lower incubation temperatures.¹⁷⁷⁻¹⁷⁸ This was demonstrated to be due to an increase in the activity of the $\Delta 12$ -desaturase enzyme catalysing the transformation of oleoyl-CoA (18:1) to linoleoyl-CoA (18:2) at lower temperatures. This effect could however be strain-specific. While not as influential as temperature, glucose concentration affected the lipid profile in the 25-30 °C range. As the glucose concentration increases, the percentage concentration of 18:1 decreases inversely with an increase in 16:0. At low nitrogen levels, there was a considerable drop in the percentage concentration of 18:1, although this variable does not have as much influence as the temperature. Increasing nutrient limitation has been previously demonstrated to increase the saturated fatty acid composition of the oil produced,⁸⁷ most likely due to metabolic overflow at excess carbon conditions resulting in the channelling of excess citrate into fatty acid biosynthesis.⁸⁶

3.2.1.4 Palmitic acid (16:0) production

In contrast to biodiesel, producing a palm oil replacement suitable to the care products or food industry requires an enhanced saturated component. Typically, palm oil contains between 35 and 50% saturated components.¹⁶ The level of palmitic acid produced by *R. glutinis* is heavily dependent on all the environmental variables. Similarly to 18:1 production, the levels of 16:0 was found to be reliant on

temperature, with high levels produced at 25 °C. A similar relationship between temperature and the degree of unsaturation within the resulting oil was also found in *C. curvatus*, in which the total saturated fatty acid content increased from 43 wt% at 34 °C to 54.2 wt% at 22 °C.¹⁷⁵ In contrast, research surrounding the production of polyunsaturated fatty acids from *R. glutinis* YM25079 showed that decreasing temperature from 25 °C to 15 °C increased the levels of linoleic and linolenic acid (18:2 and 18:3, respectively) from 29% to 55%, due to increased activity of $\Delta 12$ -desaturase.¹⁸¹ This was also observed in the cryophilic yeast, *R. glacialis*, in which the levels of saturated lipids (C16:0 and C18:0) decreased with a decrease in growth temperature.⁴⁸

At high glucose or low nitrogen levels, the palmitic acid component of the glyceride lipids reached as high as 30%. The C/N ratio did not have a large effect on the production of palmitic acid. At high glucose concentrations, reasonably high levels of 16:0 were produced irrespective of nitrogen availability. Similarly, at low glucose concentrations, little 16:0 was formed irrespective of nitrogen availability. When low levels of 18:1 were produced, such as at low temperatures, the levels of methyl palmitate production increased substantially (Figure 3.5). The 16:0 production ranged from 17% (10 g/L glucose, 1.5 g/L (NH₄)₂SO₄, 35 °C) to 32 % (30 g/L glucose, 0.5 g/L (NH₄)₂SO₄, 25 °C). Though the 16:0 content can be increased further, up to 40%, with high glucose levels. This level of saturation is comparable to palm oil. Interestingly, this large effect of temperature on the lipid profile is not generally observed in other oleaginous yeast. Suutari *et al.* showed that increasing the growth temperature either had little effect on, or increased the relative proportion of 16:0 for the yeast *S. cerevisiae*, *R. toruloides*, *C. utilis*, *L. starkeyi* and *C. oleophila*. However, when looking at the ratio between C₁₆ and C₁₈ fatty acids, the proportion of C₁₆ FAME was shown to increase at decreasing temperatures for *S. cerevisiae*.¹⁷⁴ As seen previously in this study, the concentration of nitrogen seemed to have little effect on the levels of 16:0 (Figure 3.5), however high glucose concentrations drastically improved 16:0 FAME production. An increase of 40% for the production of 16:0 was observed between 10 g/L glucose (18.2% 16:0) and 30 g/L glucose (29.8% 16:0) at 25 °C and 1 g/L (NH₄)₂SO₄ (From this, the coefficient of

determination (R^2) of the model for Y_1 , Y_2 , Y_3 and Y_4 were 0.979, 0.987, 1 and 0.999, respectively, indicating that the model explains between 97.9 – 100% of the variability in the response. The root mean squared errors (RMSE) of the models for Y_1 , Y_2 , Y_3 and Y_4 were 0.06 g/L, 1.868% d.wt., 0.632% and 0.487%, respectively, with the predicted sum square error (PRESS) RMSE, a measure of the RMSE calculated after the omission of each data point sequentially to provide an indication of model over-fitting, was acceptable with values of 0.069 g/L, 2.548% d.wt., 1.169% and 0.64% respectively (Table 3.2).

3.2.1.5 Validation of the model

In order to assess the accuracy of the model for predictive purposes, a number of validation tests were undertaken within the mid-points of the levels used for the RSM and compared to the values predicted by the models. These validation cultures were performed at 28 °C with: (1) 15 g/L glucose, 0.75 g/L $(\text{NH}_4)_2\text{SO}_4$; (2) 25 g/L glucose, 0.75 g/L $(\text{NH}_4)_2\text{SO}_4$; (3) 15 g/L glucose, 01.25 g/L $(\text{NH}_4)_2\text{SO}_4$; (4) 25 g/L glucose, 01.25 g/L $(\text{NH}_4)_2\text{SO}_4$. In general, these experimental points fell within the range of experimental error for the model, demonstrating adequate confidence in the predictions (Figure 3.1 – square, red marker points).

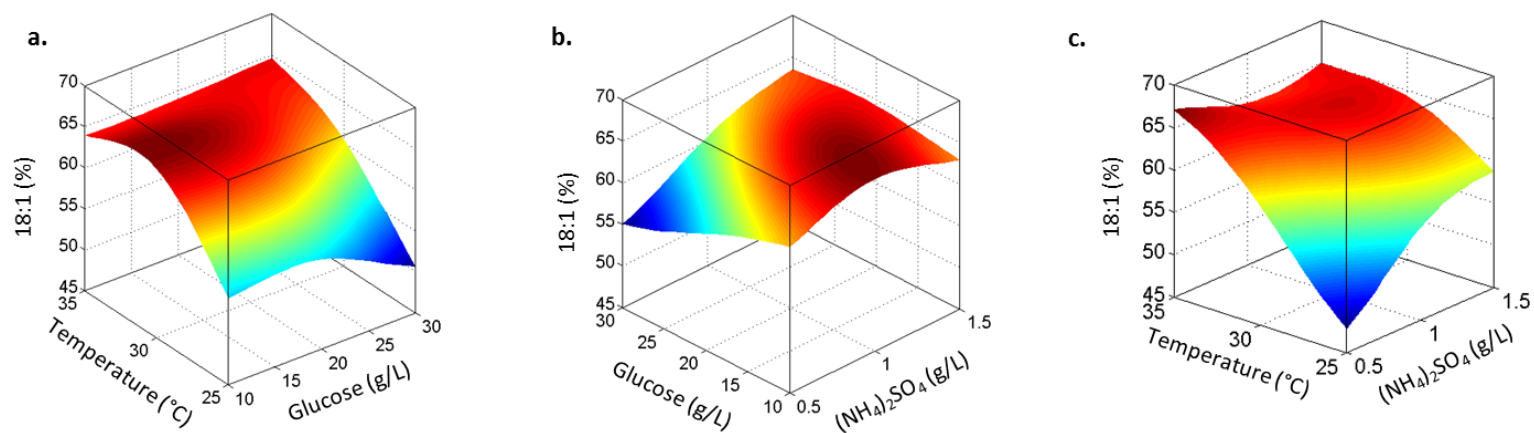


Figure 3.4: Response surface plots for the percentage of oleic acid (18:1) produced by *Rhodotorula glutinis*. a. Interaction between glucose and temperature; b. Glucose and $(\text{NH}_4)_2\text{SO}_4$; c. $(\text{NH}_4)_2\text{SO}_4$ and temperature.

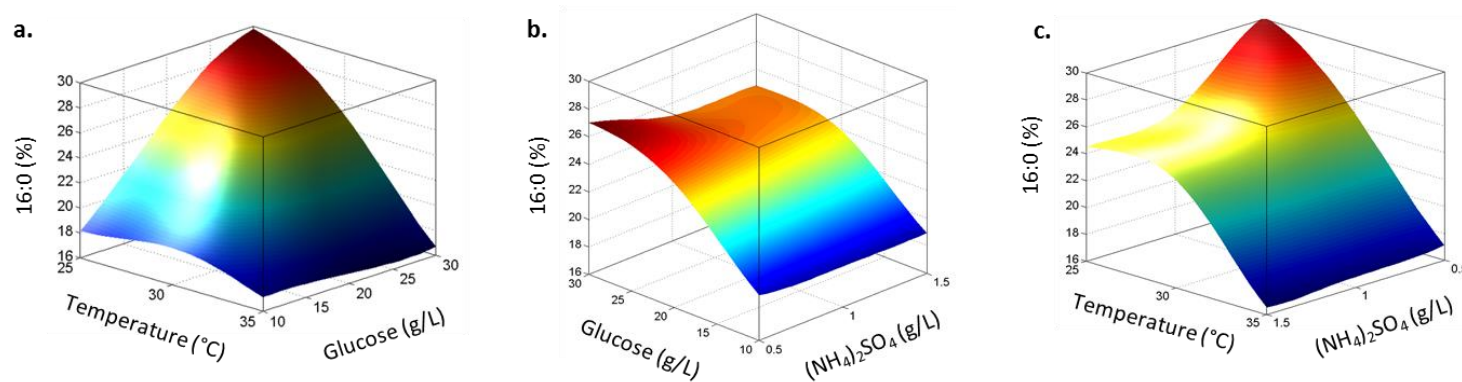


Figure 3.5: Response surface plots for the percentage of palmitic acid (16:0) produced by *Rhodotorula glutinis*. a. Interaction between glucose and temperature; b. Glucose and $(\text{NH}_4)_2\text{SO}_4$; c. $(\text{NH}_4)_2\text{SO}_4$ and temperature.

3.2.2 Tailored lipid application

In order to assess the model as a tool for tailoring the lipid profile, *R. glutinis* was cultured under conditions predicted to produce a saturate-rich oil (SRG oil) or a monounsaturated oil. These were: 30 g/L glucose, 0.75 g/L (NH₄)₂SO₄, 30 °C; and 30 g/L glucose, 0.5 g/L (NH₄)₂SO₄, 25 °C for high 18:1 and high 16:0, respectively. The monounsaturated oil was transesterified with methanol to produce biodiesel (*R. glutinis* methyl ester; RGME). The pertinent physical properties (lipid profile, density, kinematic viscosity and cloud point) were measured and compared to palm oil and rapeseed methyl ester (Table 3.3).

Table 3.3: Comparison of the properties of an oleic acid ester rich biodiesel produced from *Rhodotorula glutinis* (*R. glutinis* methyl ester) and an alternative oil, rich in saturated esters, produced by *R. glutinis* (saturated *R. glutinis* oil).

Properties	RGME (high in 18:1, %)	Rapeseed methyl ester [†] (%)	SRG oil (high in 16:0, %)	Palm oil [†] (%)
16:0	17	2 – 5	31	39 – 50
18:0	3	0 – 4	5	3 – 5
18:1	67	51 – 68	46	38 – 45
18:2	10	18 – 25	11	8 – 12
18:3	2	7 – 11	4	Trace
Density (kg m ⁻³)	933	860 – 900	879.2	908 [‡]
Kinematic viscosity (mm ² s ⁻¹ , 40 °C)	3.46	4.2 – 4.8	37.3	36 [‡]
Cloud point (°C)	-1.5	-5 - 1	Room temp.	12 [‡]

[†]The ranges typical for rapeseed and palm oil fatty acid profiles, and RME physical properties were taken from one source,¹⁶ though they are based on 44 separate scientific publications.

[‡]Typical values for palm oil taken from two sources.^{182,183}

RGME: *R. glutinis* methyl ester; RME: Rapeseed methyl ester; SRG: Saturated *R. glutinis*.

3.2.2.1 Suitability of *R. glutinis* oil as a biodiesel feedstock

While the saturated component of the RGME, of 20%, was higher than that generally found in rapeseed methyl ester (RME) samples, the cloud point remained comfortably within the range presented in the literature.⁴ The kinematic viscosity, which is also affected by a high saturated component, was well within the range for most biofuels, lower than typical RME values and comparable with EN 14-214 restrictions. One crucial difference between RGME and RME was the polyunsaturated components. Due to the increase in the quantity of reactive double bonds, polyunsaturates are far more prone to oxidation than monounsaturated or saturated esters.¹⁸⁴ Due to the differing levels of antioxidant in

the samples, it is generally difficult to compare fuels from different sources, but biofuels with a higher saturated component are generally more stable. The polyunsaturated component of RGME was found to be 12%, considerably lower than that generally observed in RME, which further demonstrates the suitability of this source as a fuel substitute. This is especially applicable when assessing only the linolenic acid (18:3) component of the oil, which is limited to 12 mol.% (~12 wt.%) in biodiesel according to EN 14-214 specification. Rapeseed oil just fits within this specification. RGME in contrast, is comfortably under this threshold, containing only 2% (w/w) linolenic acid. Furthermore, the high oleic acid content of the oil would be highly beneficial for oleochemistry. Unpublished data by Jenkins *et al.* has demonstrated that the cross metathesis of *R. glutinis* oil with ethene at 10 bar, using Hoveyda-Grubbs 2nd generation catalyst (5 mol.%) at 60 °C yielded 3.9% 1-decene as calculated by GC-MS. This would be an ideal uni-molecular fuel for the aviation industry. A range of short chain fatty acids and alkenes were also produced which are proposed to be a suitable drop-in fuel for the road transport industry.¹⁸⁵

3.2.2.2 *R. glutinis* oil as a palm oil substitute

Approximately 85% of all palm oil is produced in Indonesia and Malaysia, where accelerating demand is contributing to a 1.5% annual rate of deforestation of tropical rainforests.¹⁸⁶ Not only does this cause significant ecological damage, but it is also associated with large GHG emissions. Of the 167 million tonnes produced in 2013, 83% of the palm oil produced worldwide was used as a component in foodstuffs such as chocolate, and 4% used in care products.¹⁸⁷ Both of these applications require a high cloud point and a high viscosity. The saturate rich oil (SRG) was found to have lower overall saturates than generally found in palm oil (36% compared to ~49%). However, as there was also less polyunsaturates in the SRG oil, the viscosity was slightly higher than the typical palm oil, demonstrating the suitability of the SRG oil as a replacement for palm oil in these applications. Fargione *et al.* estimated that by producing oil from lignocellulose feedstocks, instead of converting a hectare of rainforest to palm oil cultivation, would save 1294 tonnes of CO₂; a carbon debt that would take 423 years to pay back.¹⁸⁸ A key

sustainability goal is therefore to replace lipids necessary to the fuel and care product industries with renewable alternatives sourced from lignocellulose.

3.2.3 Tailoring the lipid profile of *R. minuta*

In contrast to *R. glutinis*, *R. minuta* has been relatively unexplored from both a fundamental and applied perspective. When growing *R. minuta* on glucose as the carbon substrate, Saxena *et al.* demonstrated that *R. minuta* could achieve a lipid content of 48% (w/w), with a 22.5% lipid co-efficient yield (g lipid/ g glucose).⁸¹ Most notably, they reported that the lipid contained fatty acids within the C₇ - C₁₈ range. This is of particular interest to lipid feedstocks for the transportation industry. These short-medium chain fatty acids would offer enhanced cold flow properties, as both the melting point and viscosity decrease with a decreasing chain length.⁷⁵ As such, the same response surface methodology used to assess *R. glutinis* was applied to *R. minuta* in which glucose concentration, (NH₄)₂SO₄ concentration and temperature were varied. The 30 experiments conducted, along with three repeats of the central point in order to assess experimental variance are shown in Table 3.4.

A regression analysis was carried out to fit the response function and predict the outcomes using a radial basis function-multiquadratic (qRBF) response surface. The model fit data is shown in Figure 3.6.

Table 3.4: Experimental range for the three independent variables used in response surface methodology for *Rhodotorula minuta* in terms of coded and actual factors, and experimental data for the response surface analysis.

Test	Independent Variables			Dependent Variables			
	Temp.	Glucose	(NH ₄) ₂ SO ₄	Biomass	Total lipid	Biomass	Lipid
	(°C)	(g/L)	(g/L)	(g/L)	(% d.wt)	coefficient (%)	coefficient (%)
	x_1	x_2	x_3	Y_1	Y_2	Y_3	Y_4
1	-1(25)	-1(10)	-1(0.5)	4.017	15.39	40.38	6.22
2	0(30)	-1(10)	-1(0.5)	3.736	30.92	37.36	11.55
3	1(35)	-1(10)	-1(0.5)	3.241	15.69	33.89	5.32
4	-1(25)	0(20)	-1(0.5)	7.921	24.86	39.61	9.85
5	0(30)	0(20)	-1(0.5)	7.922	19.12	36.50	6.98
6	1(35)	0(20)	-1(0.5)	3.280	9.22	34.31	3.16
7	-1(25)	1(30)	-1(0.5)	7.428	21.09	38.44	8.11
8	0(30)	1(30)	-1(0.5)	6.782	35.71	28.65	10.23
9	1(35)	1(30)	-1(0.5)	3.034	10.82	33.72	10.99
10	-1(25)	-1(10)	0(1.0)	4.004	19.22	40.14	7.71
11	0(30)	-1(10)	0(1.0)	3.688	25.39	36.88	9.36
12	1(35)	-1(10)	0(1.0)	2.795	16.46	30.53	5.02
13	-1(25)	0(20)	0(1.0)	7.427	26.75	37.23	9.96
14	0(30)	0(20)	0(1.0)	6.791	29.79	38.23	11.39
15	1(35)	0(20)	0(1.0)	3.103	17.23	32.68	5.63
16	-1(25)	1(30)	0(1.0)	8.403	30.05	37.90	11.39
17	0(30)	1(30)	0(1.0)	6.635	28.66	35.20	10.09
18	1(35)	1(30)	0(1.0)	3.441	16.57	34.76	5.76
19	-1(25)	-1(10)	1(1.5)	4.166	20.92	41.67	8.72
20	0(30)	-1(10)	1(1.5)	3.797	24.53	38.09	9.35
21	1(35)	-1(10)	1(1.5)	2.854	13.20	31.35	4.14
22	-1(25)	0(20)	1(1.5)	7.405	25.71	ND	ND
23	0(30)	0(20)	1(1.5)	6.184	21.10	30.99	6.54
24	1(35)	0(20)	1(1.5)	3.149	14.51	32.85	4.77
25	-1(25)	1(30)	1(1.5)	8.640	28.43	40.03	11.38
26	0(30)	1(30)	1(1.5)	6.071	22.69	28.81	6.54
27	1(35)	1(30)	1(1.5)	3.062	19.16	29.80	5.71
28	0(30)	0(20)	0(1.0)	6.499	24.60	32.86	8.08
29	0(30)	0(20)	0(1.0)	6.130	21.93	30.69	6.73
30	0(30)	0(20)	0(1.0)	7.087	26.25	37.98	9.97

For independent variables, the value given is that used in the model and values in parentheses are the true values. Dwt: Dried weight; x: independent variables; Y: dependent variables; ND: No data.

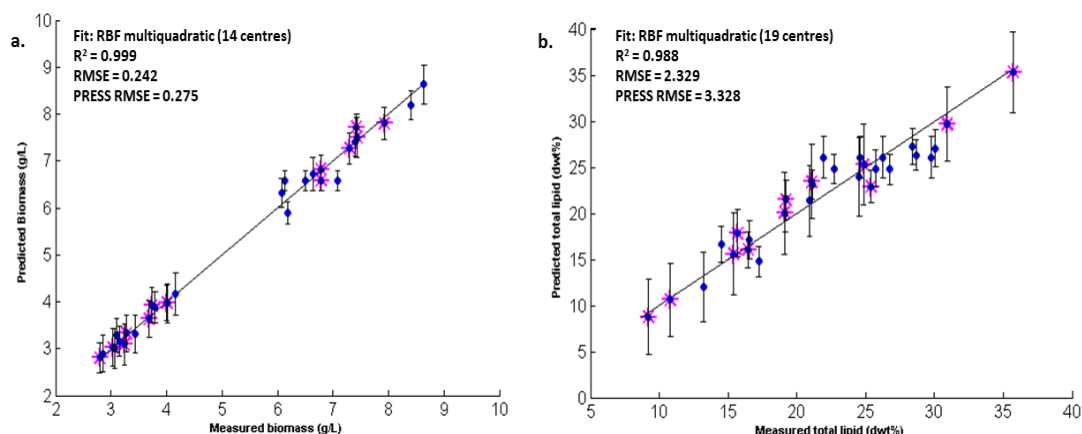


Figure 3.6: Response surface model fits for the total biomass and lipid content of *Rhodotorula minuta*. **a.** Biomass; **b.** total lipid. The predicted values represent the values obtained from the regression analysis using a radial basis function multi-quadratic response surface. The centres used in the construction of these models are indicated with a magenta star around the data point. Error bars represent the spread of data observed for repeated central point. Dwt: Dried weight; PRESS: Predicted sum square error; R^2 : Coefficient of determination; RBF: Radial basis function; RMSE: Root mean square error.

The coefficient of determination (R^2) was 0.999 and 0.988 for biomass and total lipid, respectively, indicating that 98.8 – 99.9% of the variability in the response can be explained by the model. The root mean squared error (RMSE) was 0.242 g/L and 2.329%, with the predicted sum square error (PRESS) RMSE calculated to be 0.275 g/L and 3.328% for biomass and total lipid, respectively. The PRESS RMSE is an indication of model-overfitting, on the basis of fitting the model to $n-1$ runs and taking a prediction for the remaining data (Table 3.5). Similarity between the RMSE and PRESS RMSE values for biomass and lipid yields indicated reliability in the fitting of the model.

Table 3.5: Analysis of variance (ANOVA) for the dependent variables of the response surface-guided experiments for *Rhodotorula minuta*.

	Source	SS	DF	MS	R^2	RMSE
Y_1 Biomass	Model	945.08	13.905	67.969	0.999	0.242
	Error	0.946	16.095	0.059		
	Total	946.408	30	0		
Y_2 Total lipid	Model	15340.385	11.55	1328.225	0.988	2.329
	Error	100.039	18.45	5.422		
	Total	15523.437	30	0		

SS, Sum of squares; DF, Degree of Freedom; MS, Mean square; RMSE, Root mean-square error; CV, Coefficient of variance.

3.2.3.1 Biomass and lipid yield

The production of biomass was seen to be largely independent of the level of nitrogen in the culture medium. The maximum biomass yield was achieved between 25 - 30 °C, with a glucose loading of over 20 g/L (Figure 3.7). This yielded approximately 8 g/L of dry biomass, lower than that achieved by Saxena *et al.* who reported a maximum biomass concentration of 15 g/L for *R. minuta*.⁸¹ Low levels of glucose loading (10 g/L) and high temperature (35 °C) were most detrimental to biomass yields. The biomass coefficients, (gram of biomass produced per gram of carbon substrate), ranged from 28.81% to 41.67%, with the highest efficiencies achieved when *R. minuta* was grown at 25 °C (Table 3.4). This means that approximately 35% of the starting substrate resulted in the final product stream. While biomass production is important, in order to provide the structure to house the lipid, it is important not to place too much emphasis on this metric.

The total amount of lipid accumulated in *R. minuta* was heavily dependent on a range of environmental factors (Figure 3.8). The highest level of lipid accumulation, 37% of the dry cell weight, was observed at a high C/N ratio with 30 g/L of glucose and 0.5 g/L (NH₄)₂SO₄ (C/N ratio of 132).¹⁷⁷ While generally a high C/N ratio has been reported to induce high lipid yields,^{17,76} unusually, a C/N ratio of just 30 was most favourable for lipid accumulation in the study by Saxena *et al.* with *R. minuta*.⁸¹ The relationship between the lipid production and temperature, however, was more complex. At high temperatures, low lipid yields were observed irrespective of other conditions, though there was little difference at temperatures of 25 °C and 30 °C. However, at 35 °C, lipid production in *R. minuta* dropped to as low as 5%, irrespective of the C/N ratio. The very low biomass and lipid yields observed at higher temperatures show the importance of controlling temperature in an industrial bioreactor system.

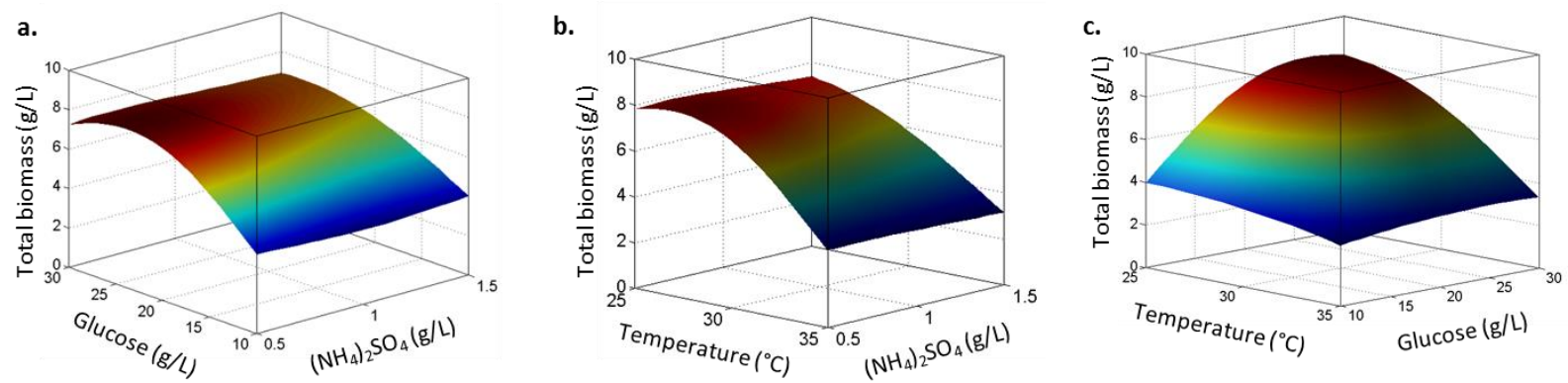


Figure 3.7: Response surface plots for the total biomass produced by *Rhodotorula minuta* a. Interaction between glucose and $(\text{NH}_4)_2\text{SO}_4$; b. $(\text{NH}_4)_2\text{SO}_4$ and temperature; c. glucose and temperature.

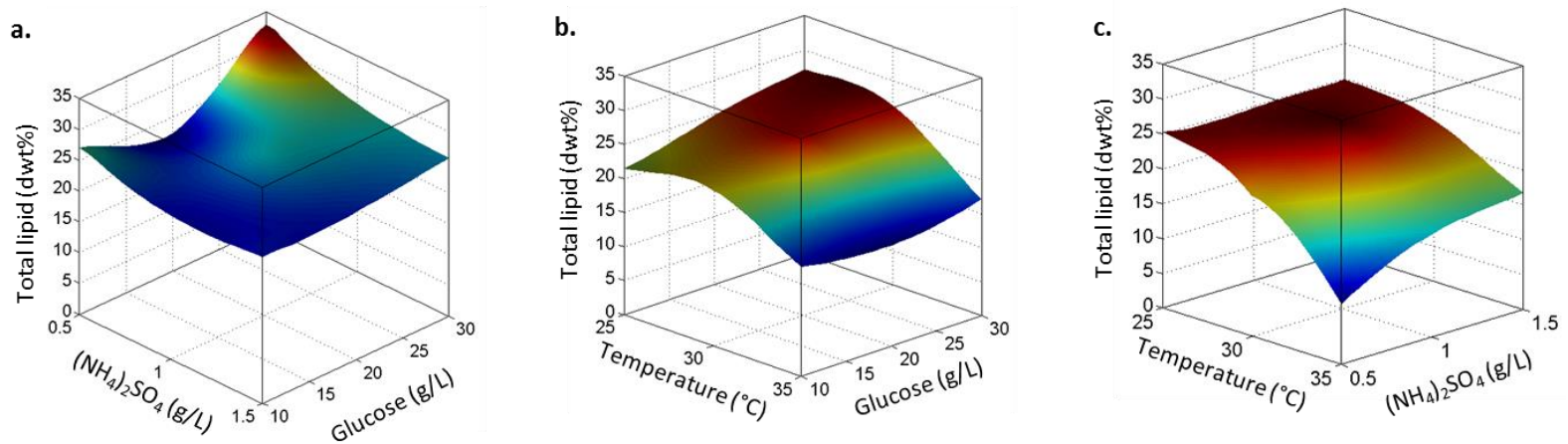


Figure 3.8: Response surface plots for the total lipid produced by *Rhodotorula minuta* a. Interaction between glucose and $(\text{NH}_4)_2\text{SO}_4$; b. glucose and temperature; c. $(\text{NH}_4)_2\text{SO}_4$ and temperature.

In order to assess the efficiency of the system, a more suitable metric is the production of lipid as a function of sugar consumption (g lipid per gram of sugar consumed), also known as the lipid coefficient. The lipid coefficient ranged between 3.2 - 11.6%. These values were comparable to those reported by Pan *et al.*, who reported a lipid coefficient of between 7.39 - 8.46% for *R. minuta* cultivated on xylose.⁴⁶ They are however slightly lower than lipid coefficients obtained for other oleaginous yeast cultivated on glucose. For example, lipid coefficients of 13% were obtained for *R. grammis*,⁵⁶ and *C. curvatus*.¹⁸⁹ Similarly, a lipid coefficient of 20% was achieved for *T. cutaneum* grown solely on glucose which decreased to 16% when xylose was used as the sole carbon source.¹⁹⁰ Using a fed-batch system, the lipid coefficient for *R. toruloides* was as high as 24%, achieving a lipid content of 60.4% (w/w) or 61.4 g/L.¹⁸⁹ While the theoretical lipid coefficient is 32%,⁹⁶ even under ideal growth conditions it is rarely more than 22% due to other metabolic processes requiring glucose.²² It is because of this that Ratledge & Cohen suggested that 40% (w/w) was a more realistic target for SCO production.³⁵

3.2.3.2 *R. minuta* lipid profile

Most surprisingly, the fatty acid profile of *R. minuta* did not change significantly on changing the environmental conditions (Figure 3.9). The fatty acid profile consisted of four major fatty acids, with approximately 18% palmitic acid (16:0), 4% steric acid (18:0), 62% oleic acid (18:1) and 16% linoleic acid (18:2). This fatty acid composition is highly similar to rapeseed oil, and would, upon transesterification, fall within the European standard for biodiesel production.¹⁶ In contrast, Saxena *et al.* reported a change in the lipid profile of *R. minuta* with changes to the growth temperature. They did however report C₁₆ and C₁₈ lipids to be most prevalent at 30 - 32 °C, whereas short-chain fatty acids (C₇-C₉) were observed at 38 °C, and no alteration to the levels of C₁₀-C₁₄ lipids as a function of temperature.⁸¹ Within this study, no fatty acids shorter than C₁₆ were observed through GC-MS analysis, suggesting that different strains may have a large contribution to the resulting fatty acid profile.

The stability of the fatty acid profile upon changing environmental conditions has advantages from a biotechnology perspective. Stabilising the temperature in industrial fermentations is a major cost, and thus the ability to produce high levels

of oil at varying temperatures is of benefit. In order for the process to be industrially viable, low-cost, renewable feedstocks must be used. However, when using multi-variant feedstocks such as depolymerised lignocellulose or waste streams, the input variables cannot be controlled sufficiently. By using an organism such as *R. minuta* that has a stable lipid profile, and therefore guaranteed physical properties, regardless of the feedstock will guarantee the same quality product irrespective of inputs. The use of these types of biomass streams for the growth of *Rhodotorula sp.* is explored further in Chapter 5.

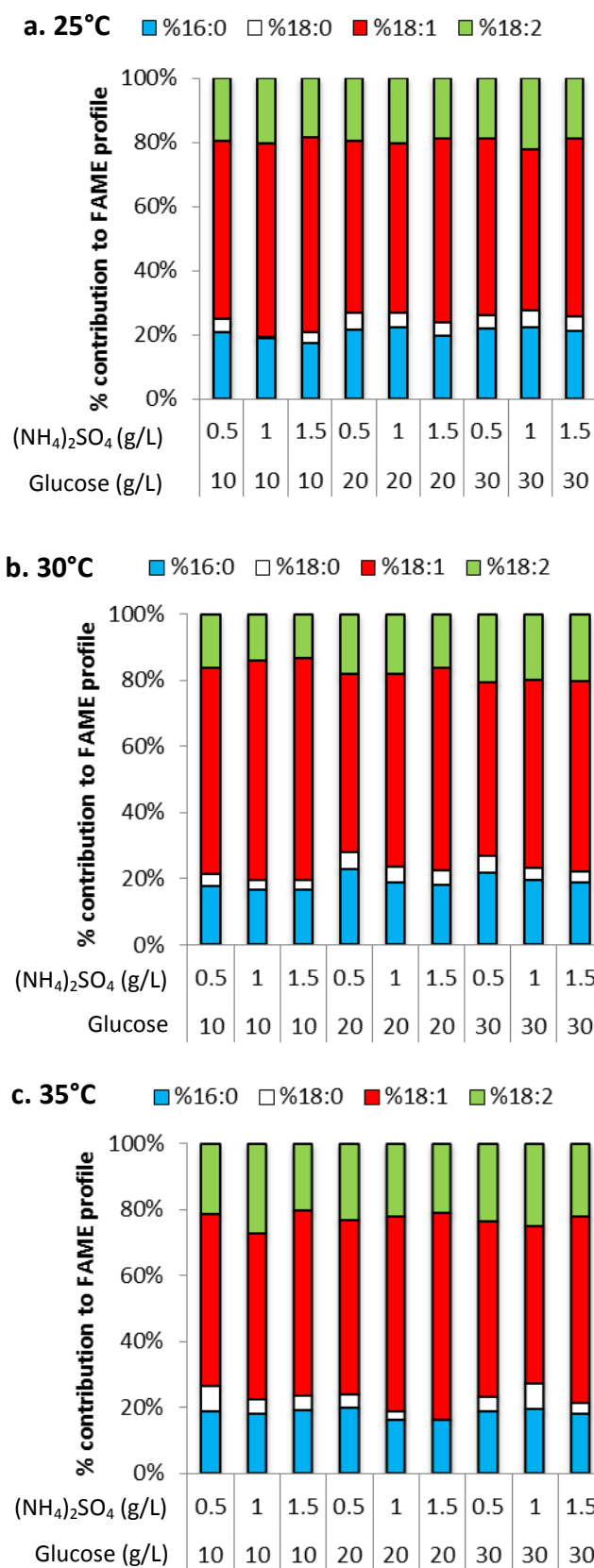


Figure 3.9: The lipid profile of *Rhodotorula minuta* cultivated at: a. 25 °C; b. 30 °C; and c. 35 °C. The concentration of glucose and (NH₄)₂SO₄ used to supplement the RSM medium in the culture conditions is also presented. The colours represent the relative percentage of lipid types: Blue (%16:0); white (%18:0); red (%18:1); green (%18:2).

3.2.4 Conclusion

The work presented in this chapter has used a Design of Experiments in order to optimise the lipid production for two oleaginous yeasts; *R. glutinis* and *R. minuta*. For *R. glutinis*, high culture temperature and high nitrogen ratio yielded a mainly monounsaturated oil, while low temperatures and high glucose loadings gave a more saturated profile. Upon transesterification, the oil high in monounsaturated esters yielded biodiesel with fuel properties akin to rapeseed methyl ester, whereas the oil high in saturates was found to be a suitable replacement for palm oil. When the same methodology was applied to *R. minuta*, lower cultivation temperatures (25-30 °C) and high levels of glucose loadings were found to be most beneficial to biomass and lipid productivity. While previously published data suggested that the lipid profile of *R. minuta* varied with changing environmental conditions,⁸¹ to include the synthesis of C₇ – C₁₈ lipids, no evidence of this was observed in this study. The fatty acid profile of *R. minuta* was found to be stable irrespective of the environmental conditions. This would be highly beneficial when transitioning to waste resources, where the composition of the input stream cannot be guaranteed. The lipid profile does however suggest that upon transesterification, the resulting biodiesel would be ideal as a replacement for petrodiesel.

CHAPTER 4:
THE EFFECT OF SONICATION ON THE LIPID ACCUMULATION
IN *RHODOTORULA SP.*

“Don’t reinvent the wheel, just realign it.”

Anthony J. D’Angelo

4.1 Preamble

In this chapter, the optimised growth conditions presented in Chapter 3 are explored from an industrial perspective to enhance lipid yield. This work was conducted in collaboration with Almac Group, N. Ireland and has been submitted for publication to the journal *Sustainability* (under review).

4.2 Introduction

Energy as sound waves with a frequency greater than 20 kHz is referred to as ultrasound. It is beyond the sound frequency that can be detected by the human ear and can reach frequencies of up to 10 MHz. The level of ultrasound can be broken down into three categories: low frequency, high power ultrasound (20 - 100 kHz); intermediate frequency, medium power ultrasound (100 kHz – 2 MHz); and high frequency, low power ultrasound (2 - 10MHz).¹⁹¹ Ultrasound is widely used in industrial biotechnology applications to disrupt cells and release the intracellular components. This is especially useful in the manufacture of enzymes. The mechanism of cell disruption is associated with a cavitation effect, in which microbubbles form at several nucleation sites during the rarefaction phase of the sound wave and then collapse during the compression phase releasing an intense shock that travels through the medium (Figure 4.1).¹⁹²

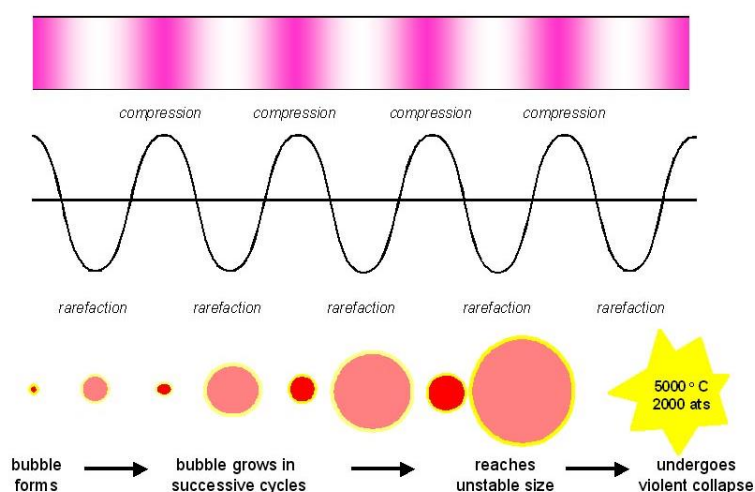


Figure 4.1: Representation of the generation and collapse of an acoustic cavitation bubble.¹⁹¹

During cavitation, the sonic energy is transferred into mechanical energy in the form of elastic waves, which can disrupt the cells present in the cavitation area. While this cavitation effect can break apart bioactive macromolecules, at lower ultrasound intensities, it can also cause inactivation of the cells.¹⁹³ Used at appropriate levels, the generation of microbubbles by sonication does however offer exciting possibilities to aid mass transfer processes within fermentations.

4.2.1 Generation of microbubbles

In a bioreactor, the microbial cells are surrounded by a film of static liquid which can impede the mass transfer of gases, substrates and nutrients into the cell.¹⁹³ In continuous stirred tank reactors (CSTR) high gas and mass transfer rates are achieved through the use of impellers or an increased gas flow rate, but the elevated sheer stress can be detrimental to some microorganisms.¹⁹⁴ Compared to other soluble nutrients such as glucose, ammonium and most salts, the solubility of oxygen in water is relatively low (0.217 mM at 35 °C in pure water), but the utilisation rate is high.¹⁹⁴ The solubility of oxygen in water also decreases in the presence of salts required for growth, further exacerbating the problem.¹⁹⁵ Oxygen transport is roughly proportional to the surface area to volume ratio of the bubble; i.e., the smaller the bubble, the higher rate of gas transfer.¹⁹⁵ Bubbles generated by a conventional sparger are generally in the order of 3-5 mm in diameter.¹⁹⁴ Previous published work has suggested that the mass transfer rate of oxygen in fermenters was increased when sparged with a microbubble dispersion generator (MBD).¹⁹⁴⁻¹⁹⁶ The generation of microbubbles (20 - 1000 μm) can also be achieved through the use of sonication. The microbubbles formed within the bioreactor can have a microstreaming effect, in which small-scale eddying is generated that can thin the layer around the cells.¹⁹⁷ This thinning of the film around the cells can in turn enhance the transfer of growth nutrients and gases through the fluid layer and into the cell. Furthermore, gas transfer is increased, both with the removal of CO_2 as well as the increase in dissolved oxygen within the growth medium.¹⁹³ This is because smaller bubbles have an increased residence time in the fermenter, increasing oxygen delivery to the fermenting yeast.¹⁹⁵ This is particularly important

in aerobic fermentations, in which oxygen can be essentially considered as a nutrient.

4.2.2 Ultrasound-assisted fermentation

The majority of research into beneficial ultrasound fermentations has been undertaken on the ethanol producing yeast, *Saccharomyces cerevisiae*. Continuous sonication at 1 MHz (10.5 W cm^{-2}) was found to be inhibitory to *S. cerevisiae*.¹⁹⁸ However, continuous, low power ultrasonication (300 W m^{-2} , 43 kHz) with various strains of *S. cerevisiae* decreased the fermentation time of beer, wine and sake production by 50 – 64%.¹⁹⁹ This effect was thought to be due to the decreased levels of the inhibitory dissolved CO_2 within the fermentation broth, possibly though enhanced degassing.¹⁹⁹ Lower energy ultrasonication provided intermittently for a short duration has also been shown to be beneficial to the productivity of bioprocesses involving live cells. Low-intensity (118 W cm^{-2}) intermittent ultrasound of *S. cerevisiae* at 20% (1 s sonication followed by 5 s of no sonication) was found to enhance ethanol yields by 3.5-fold compared to the control.²⁰⁰ Several studies have documented that sonication of the fermentation broth through continuous recirculation between a stirred tank bioreactor and an externally located ultrasonic chamber can affect the fermentation conditions. For example, intermittent sonication with a 25 kHz tube resonator, with a continuous output of 80 W resulted in a 76% increase in the release of intracellular gentamycin from the bacterium, *Micromonospora echinospora*.²⁰¹ While numerous studies have investigated the effect of sonication on the biomass and ethanol production in yeast,^{200,202} no studies have detailed the effect on lipid accumulation in oleaginous organisms.

4.2.3 Effect of oxygen on oleaginous yeast

The issues surrounding oxygen mass transfer limitation mentioned previously are particularly heightened when aerobic yeast cultures, such as *R. glutinis*, are cultivated. For oleaginous yeast there is however a balance between lipid production and the production of the biomass that houses the lipid, as the same substrate is used for both processes.³⁵ Biomass yields of 185 g/L were achieved when *R. glutinis* was cultivated with oxygen-enriched air, but lipid concentrations of

40% (w/w) could only be achieved through nitrogen limitation.⁷⁴ When assessing solely dissolved oxygen (DO), changes in the DO levels have been shown to effect the total lipid accumulation in oleaginous yeast. Lipid accumulation in *C. lipolytica* was favoured at low levels of dissolved oxygen,²⁰³ whereas Choi *et al.* demonstrated that high levels of dissolved oxygen (DO) were beneficial to lipid accumulation in *R. gracilis*.²⁰⁴ For *R. glutinis*, low DO levels resulted in decreased biomass, but high lipid accumulation. However, even though the cellular lipid content was 10% lower in the high DO cultures, the maximum biomass yield increased by 17 g/L compared to the low DO conditions, and thus more lipid was produced by the system overall.²⁰⁵

4.2.4 Aim of the chapter

While sonication has been used to enhance ethanol production in yeast, the effect of sonication on oleaginous yeast has not been explored. This chapter therefore aims to transfer the results of the shake-flask experiment in Chapter 3, to 2.5 litre fermenters. Firstly, the effect of pH control on the biomass and lipid productivity of *R. glutinis* will be understood, before testing the effects of a variety of sonication regimes on *R. glutinis* and *R. minuta*, using a sonication rig designed and constructed in collaboration with Almac Group and Celbius Ltd.

4.3 Results and discussion

In order to achieve the maximum lipid production from the system, the RSM medium (described in Section 2.1.3), supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ was used for all of the resulting fermentations (C/N ratio of 132). According to the shake flask experiments performed in the previous chapter, the expected biomass and lipid productivities are shown in Table 4.1.

Table 4.1: Biomass and lipid productivities achieved for *Rhodotorula glutinis* and *Rhodotorula minuta* when grown in shake flasks containing RSM medium supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ for 120 h at 180 rpm.

	<i>R. glutinis</i>		<i>R. minuta</i>	
	25°C	30°C	25°C	30°C
Biomass (g/L)	8.174	8.299	7.428	6.782
Total Lipid (% d.wt)	29.24	31.76	21.09	35.71
Lipid Profile (wt. %)				
Palmitic acid (16:0)	31.42	26.91	21.92	21.97
Palmitoleic acid (16:1)	1.61	1.55	0	0
Steric acid (18:0)	5.33	5.44	4.07	4.94
Oleic acid (18:1)	46.46	54.53	55.08	52.68
Linoleic acid (18:2)	11.23	9.80	18.93	20.41
Linolenic acid (18:3)	3.95	1.77	0	0

For this study, a 2.5 litre fermenter (1.5 litre working volume) was used. For the fermentations with ultrasound, the fermentation broth was circulated through a cell held in an ultrasound bath which was run continuously at 45 kHz, 0.51 W cm^{-2} (Figure 4.2). At the set flow rate, the residence time within the sonication cell was 10 s, and 90 s circulating in the rest of the system.

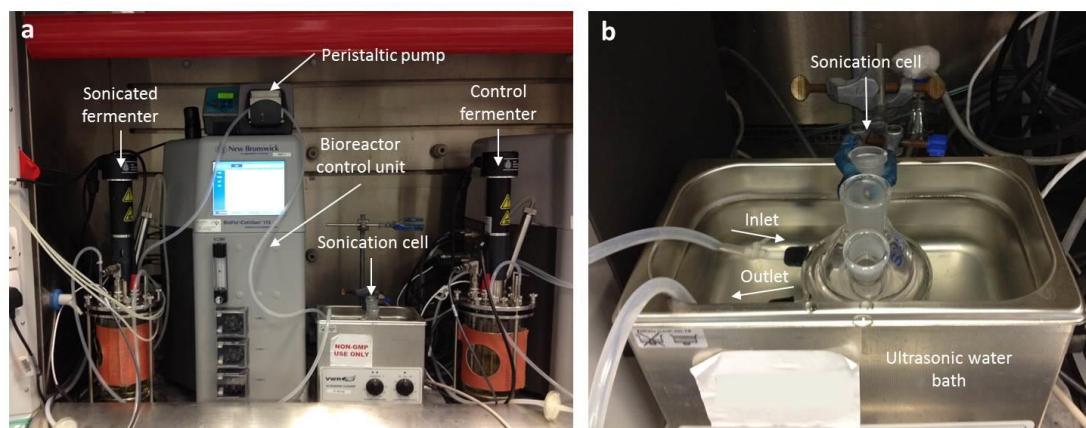


Figure 4.2: Ultrasound-assisted fermentation system constructed at Almac, N. Ireland, using a 2.5 L continuous stirred tank reactor. (a) Whole rig set-up. (b) Close-up of the sonication cell in the ultrasonic water bath.

4.3.1 Effect of pH control on *R. glutinis*

The pH was not controlled during the RSM shake flask experiments detailed previously. In order to understand if pH variation adversely affects the lipid production from *R. glutinis*, the first of the fermentations compared the effect of maintaining a stable pH within the fermentation broth to allowing the yeast naturally alter the pH of the fermentation medium. Yeast are known to produce lipid over a wide pH range,¹⁶⁴ and slight changes to the pH of the culture medium do not generally affect the overall productivity of the system. Minor changes to the pH of the culture medium have, however, been shown to change the lipid profile of many yeast species.²⁰⁶

Over a broad pH range the effect of pH on lipid production is species-specific.¹⁶⁴ Spotholz *et al.* demonstrated that there was little difference in the growth rate of *R. gracilis* between pH 3.5 – 6.5, with the greatest net dry weight of cells produced at pH 5.5.²⁰⁷ Similarly, a pH of 5.5 was found to be the optimum pH for carotenoid production from *R. glutinis*.⁷⁷ As such, the pH in the controlled fermenter was maintained at pH 5.5 through the automated addition of 2 M potassium hydroxide. As *R. glutinis* is an obligatory aerobe, the agitation was set at 800 rpm for both the pH- and non-pH-controlled fermentations. This was to ensure that oxygen was not a limiting factor for yeast growth.

4.3.1.1 Effect of pH control on the DO, glucose and NH_4^+ concentration

For the pH-controlled fermentation, the dissolved oxygen (DO) content changed little during the fermentation period, remaining at 100 % saturation throughout (Figure 4.3). There was a slight dip in dissolved oxygen around 24 h, coupled with undulation in the pH level. This was most likely due to the beginning of the exponential growth phase in which carbon dioxide and protons are released from the cell, acidifying the culture medium. Following this period, the pH remained stable at pH 5.5 and the DO increased slightly until it remained stable at 100% saturation.

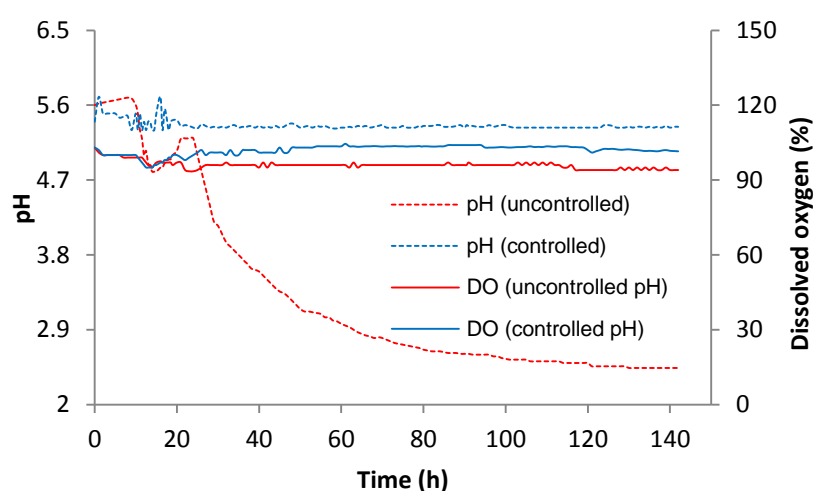


Figure 4.3: Fermentation profile for *Rhodotorula glutinis* grown under pH- and non-pH-controlled conditions. A 2.5 L fermenter with 1.5 L RSM medium supplemented with 30g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ was used. Growth occurred at 28 °C with a fixed agitation of 800 rpm for 140 h. For the pH-controlled fermentation, the pH was maintained at 5.5 through the automatic addition of 2 M KOH. The dissolved oxygen concentration was monitored but not controlled. Data was logged automatically every 15 min using Biocommand Track and Trend software. The data represents a one-off scoping study.

Similarly to the pH-controlled fermentation, in the non pH-controlled fermentation the dissolved oxygen remained relatively stable at around 96% saturation throughout, indicating that oxygen was not a limiting factor for yeast growth (Figure 4.3). During the first 10 h of the fermentation, there was little change in the pH of the culture medium, however after this point, a sharp decrease from approximately pH 5.6 to 4.6 was observed, before increasing again to pH 5.2. This increase in pH has been previously attributed to the oxidative deamination of

amino acids within the yeast extract, resulting in ammonium accumulation, diffusion and a corresponding pH change.²⁰⁸ Indeed, the ammonium content in both the pH-controlled and non-pH controlled increased within the fermentation broth, from 0.67 g/L at 0 h to 0.97 g/L and 1.04 g/L at 26 h for the pH- and non-pH-controlled fermentations, respectively (Figure 4.4). Similarly, when the fungus *Collectotrichum gloeosporioides* was cultured on 1% yeast extract, a pH increase from 4.5 to 5.8 was observed.²⁰⁹ The optimum for ammonium uptake has been reported to be between 6.0 and 6.5,²¹⁰ and thus the pH rise observed would be beneficial to ammonium assimilation.

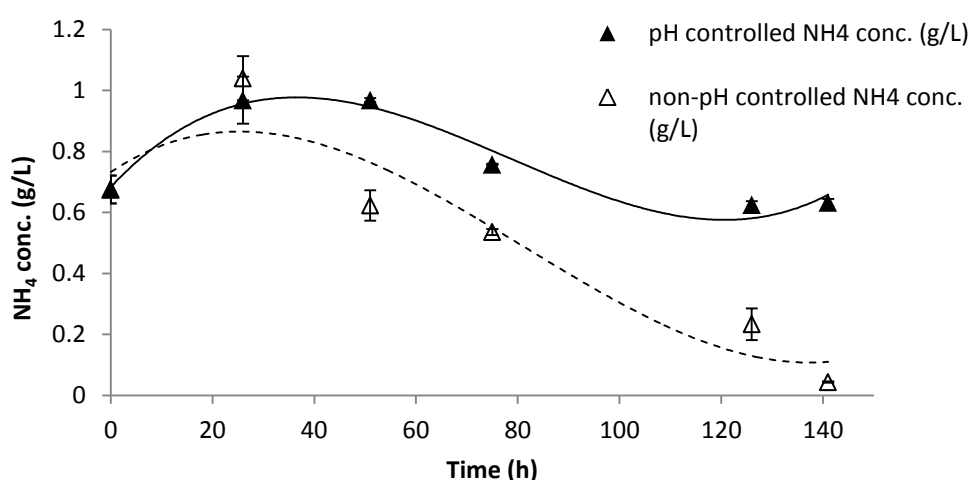


Figure 4.4: Ammonium concentration for the fermentation profile of *Rhodotorula glutinis* grown in pH (5.5) and non-pH controlled conditions. The RSM growth medium was supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ and cultures at 28 °C, with a fixed agitation of 800 rpm for 140 h. The error bars represent the standard deviation from the mean where $n=3$. The lines represent a polynomial fit using Microsoft Excel.

After 26 h, the pH continued to decrease, inversely proportional to the increase in growth of *R. glutinis* (Figure 4.5). This was also coupled to a decrease in the glucose and ammonium concentrations. As expected, the decrease observed for both of these was greater for the non-pH-controlled fermentation as the growth rate was higher and thus more carbon and nitrogen would be required for cellular metabolism and division.

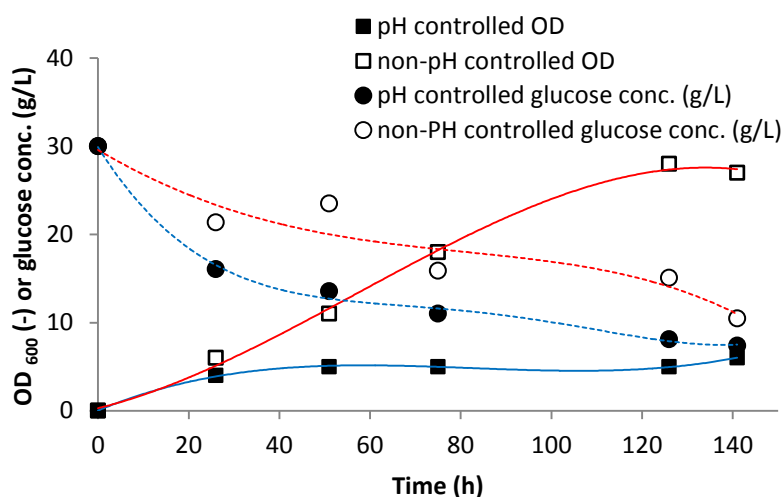


Figure 4.5: Glucose and optical density (OD) fermentation profile for *Rhodotorula glutinis* grown under pH-controlled (pH 5.5) and non-pH-controlled conditions. *R. glutinis* was inoculated into 1.5 L of the RSM medium supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$, and cultured at 28 °C, 800 rpm for 140 h. 10 ml samples were aseptically removed from the culture medium at the indicated time points and the OD measured at 600 nm. Glucose levels were quantified by HPLC. The points represent a single scoping study. The lines represent a polynomial fit using Microsoft Excel.

Acidification of the culture medium has been reported previously for *R. glutinis*. For example Leelasart & Bonaly, observed that the pH changed from 5.6 to 3.8 when culturing *R. glutinis* without controlling the pH.²¹¹ Similarly, Cho *et al.* observed a pH decrease from the starting pH of 4.0 down to a value of 1.9 during the growth of *R. glutinis*.¹⁸⁰ Under these conditions the final pH was lowest when ammonium salts were the sole nitrogen source. The authors proposed that the yeast expelled protons from the cell when the ammonium was metabolised, causing acidification of the culture medium.¹⁸⁰ Some yeast, such as the ascomycetiae yeast, *Y. lipolytica* shift away from *de-novo* lipid synthesis to extracellular citric acid production when dissolved oxygen within the culture medium is high.²⁰³ Generally, yeast that accumulate large quantities of lipids do not produce extracellular citric acid.²¹² This is especially true for basidiomycetiae yeast (such as *Rhodotorula sp.*) that do not produce organic acids. Acidification of the external medium has been linked to the uptake of metabolisable sugars. Increased activity of the plasma membrane H^+ -ATPase caused by increased levels of sugar transportation results in the expulsion of protons from the cell. Kotyl *et al.* observed that 75% of the acidification was due to the activity of H^+ -ATPase, and 25% due to the excretion of acidic metabolites

from the cells in a variety of yeast.²¹³ Furthermore, high levels of glucose in the culture medium is known to activate the plasma membrane ATPase by transcriptional and post-transcriptional modifications that increase the overall activity of ATPase in the yeast cells.²¹⁴

4.3.1.2 Effect of pH on biomass and lipid yields

The pH control had a negative effect on the growth of *R. glutinis*, as the OD for the pH-controlled fermentation reached a maximum of 6 (-) compared to 28 (-) for the non-pH-controlled fermentation (Figure 4.5). This was represented in the final biomass yields of 4.05 g/L and 6.73 g/L for the pH- and non-pH controlled fermentations, respectively (Table 4.2). These results are in contrast with Martinez *et al.* who observed no difference in the growth of *R. glutinis* using growth medium that was maintained at pH 5.2 during the fermentation period and medium that was not pH controlled, but with an initial pH of 5.2.²¹⁵ Johnson *et al.* found that the optimum pH for *R. glutinis* growth and lipid production was pH 4.0, but productivities decreased when the pH was increased or decreased from this point.²⁰⁶ When grown at pH 5.0, biomass and lipid yields were reduced by 20% and 39% compared to growth at pH 4.0.²⁰⁶ It was also found that for *R. glutinis* there was no effect on cell growth between the variable pH condition and at a constant pH of 4.5.²¹¹ This huge variation in the influence of pH on biomass and lipid productivity may therefore suggest that effects are strain-specific.

Interestingly, the total glucose consumed in the pH-controlled fermentation was greater than the non-pH-controlled fermentation, even though biomass yields were significantly lower (Figure 4.5). However, the OD remained stable at approximately 5 (-) throughout, suggesting that while not optimal for growth, *R. glutinis* was growing at a steady state. This suggests that while the yeast were not dividing, they are still respiring and thus metabolising glucose. This was also reflected in the lipid yields. The pH-controlled fermentation contained only 10.59% of the cell d.wt as lipid compared to 34.28% for the non-pH-controlled fermentation. This further substantiates that the glucose was being used for cellular processes and division than for lipid metabolism. Given this, the lipid coefficient, i.e. the lipid produced per kg of substrate was 6-fold lower for the pH-controlled fermentation. Non-optimal

growth conditions for *R. glutinis* have been shown previously to adversely affect the lipid production.¹⁶⁴ Similarly, when cultivated within a pH range of 3.45 – 5.70, biomass and lipid productivities of *Candida curvata* halved when the pH was decreased further to 2.70.²¹⁶

The biomass and lipid yield for the non pH-controlled fermentation compared favourably to the shake flask experiments. The biomass yield was slightly lower at 6.73 g/L compared to 8.2 g/L, but the intracellular lipid concentration was higher, 34% compared to 30% for the shake flask experiments (Tables 4.1 & 4.2). While the lipid profiles were similar in terms of the type of lipids produced, the relative composition of the fatty acids differed slightly. Specifically, the levels of linoleic acid (18:2) were 14.7% higher for the yeast grown in the fermenters. This was coupled to slightly lower levels of palmitic acid (16:0) and oleic acid (18:1). No palmitoleic acid (16:1) was observed in the *R. glutinis* grown in the fermenters, whereas it was present in levels up to 1.5% in the shake flask experiments.

Table 4.2: Growth and lipid productivities of *Rhodotorula glutinis* grown in pH and non-pH-controlled fermentation conditions.

	pH controlled	Uncontrolled pH
Maximum specific growth rate, μ (h^{-1})	-	0.020
Final Biomass (g L^{-1})	4.05	6.73
Biomass yield on glucose ($\text{g}_{\text{biomass}}/\text{kg}_{\text{glucose}}$)	179	345
Lipid (% dry weight)	10.59	34.28
Lipid co-efficient ($\text{g}_{\text{lipid}}/\text{kg}_{\text{glucose}}$)	19	118
Lipid profile (wt %)		
Palmitic acid (16:0)	27.9	23.9
Stearic acid (18:0)	8.4	6.5
Oleic acid (18:1)	42.6	42.7
Linoleic acid (18:2)	21.1	25.2
Linolenic acid (18:3)	0	1.7

4.3.2 Effect of ultrasonication on the growth of *R. glutinis*

While numerous studies have investigated the effect of sonication on the biomass and ethanol production in yeast,^{200,202} no studies have detailed the effect of sonication on lipid accumulation in oleaginous organisms. In the first of the sonication experiments, sonication was commenced at 42 hours after inoculation of the starting culture. The sonicated culture of *R. glutinis* was compared to a control,

which was performed under identical conditions though without sonication. The biomass productivity, glucose consumption, pH and dissolved oxygen (DO) in the batch fermenter are shown in Figures 4.6 – 4.8, respectively.

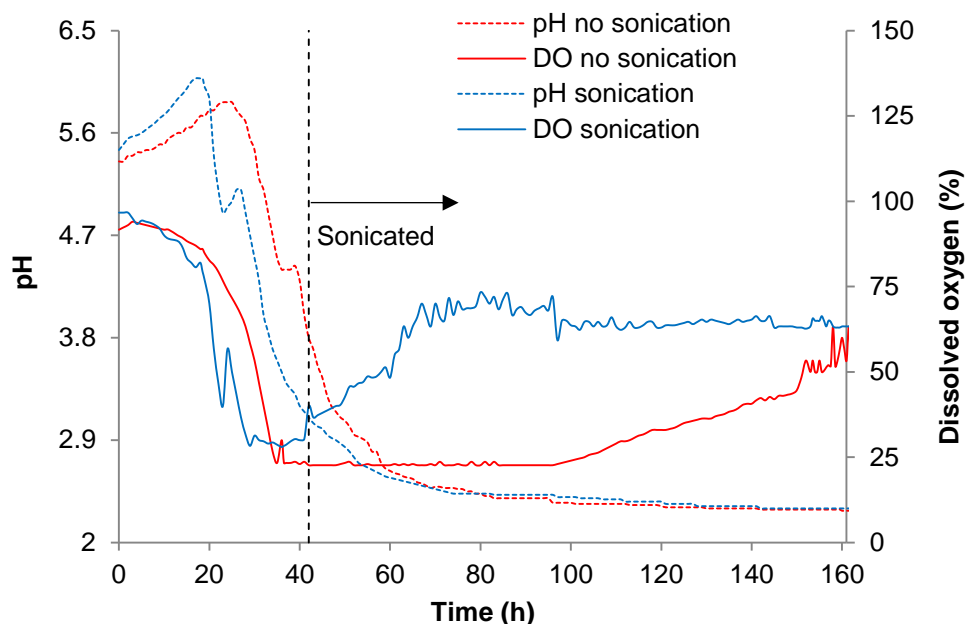


Figure 4.6: The pH and dissolved oxygen (DO) concentration for the fermentation profile for *Rhodotorula glutinis* grown with and without ultrasonication. 1.5 L of RSM medium supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ was cultured at 28 °C with cascading agitation (set point, 25%) for 160 h. Sonication commenced at 42 h. The pH was uncontrolled throughout in both conditions. Both pH and dissolved oxygen concentration were measured automatically every 15 min using Biocommand Track and Trend software. The data represents a one-off scoping study under the two conditions.

4.3.2.1 Effect of sonication on the growth profile of *R. glutinis*

Prior to sonication, the pH profile, biomass concentration and dissolved oxygen concentrations were comparable for both cultures. During optimal growth, oxygen demand within the culture is high and as such the DO profile commonly mirrors the biomass growth profile. Following inoculation, the DO remained at 100% for nearly 8 hours in both cultures, indicating that *R. glutinis* was still in the lag phase at this point. On entering the acceleration phase the DO started to drop. As the yeast entered the exponential growth phase, accelerating the demand for oxygen, the amount of O_2 in the supernatant fell to 25%, the set value for the bioreactors runs (Figure 4.25). On commencement of the sonication a spike was observed in the DO presumably due to the increased gas-liquid transfer observed on sonication.²¹⁷ From this point onwards the DO within the sonicated culture continued to rise,

suggesting that the growth of the culture was no longer optimal. The DO within the control culture remained at the 25% set point for a further 60 h before increasing. This was mirrored in the growth curves and glucose consumption (Figure 4.7). The growth curves were comparable for both cultures until commencing sonication, at which point the biomass growth for the sonicated culture decreased in comparison to the control even though there was a plentiful supply of glucose and nitrogen within the broth.

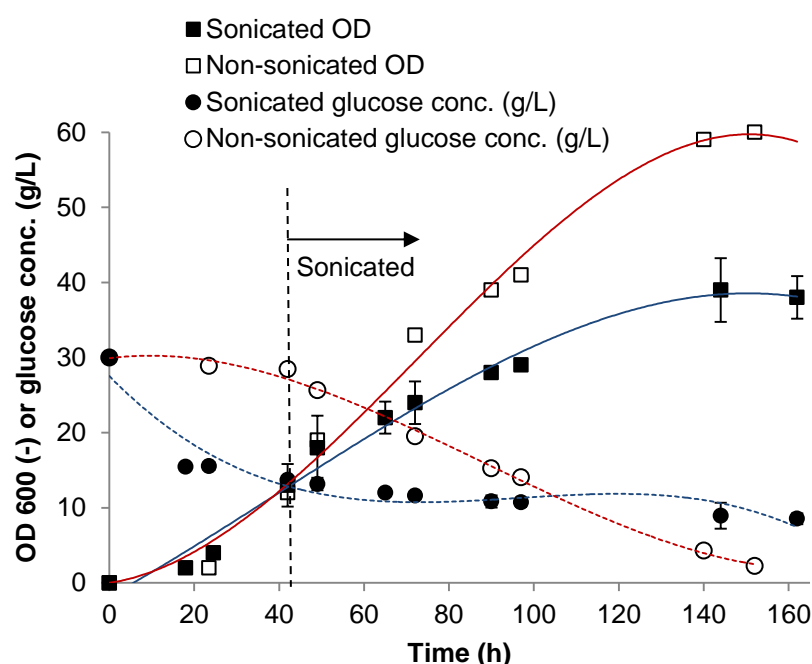


Figure 4.7: The optical density (OD) and glucose concentration for the fermentation profile for *Rhodotorula glutinis* cultured with and without ultrasonication. 1.5 L of RSM medium supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$, was cultured at 28 °C with cascading agitation (set point, 25%) for 160 h. Sonication commenced at 42 h. The pH was uncontrolled throughout in all cultures. The error bars indicate the standard deviation from the mean where $n=2$. The lines represent a polynomial fit using Microsoft Excel.

For both cultures, the nitrogen in the culture medium was consumed by 100 hours (Figure 4.8). Upon exhaustion of the nitrogen from the fermentation broth, the excess carbon can be channelled into lipid biosynthesis pathways, resulting in the accumulation of triacylglycerides as discrete lipid bodies within the cell.¹⁶⁵ While the glucose continued to be metabolised in the control culture, this was not observed in the sonicated yeast culture (Figure 4.8).

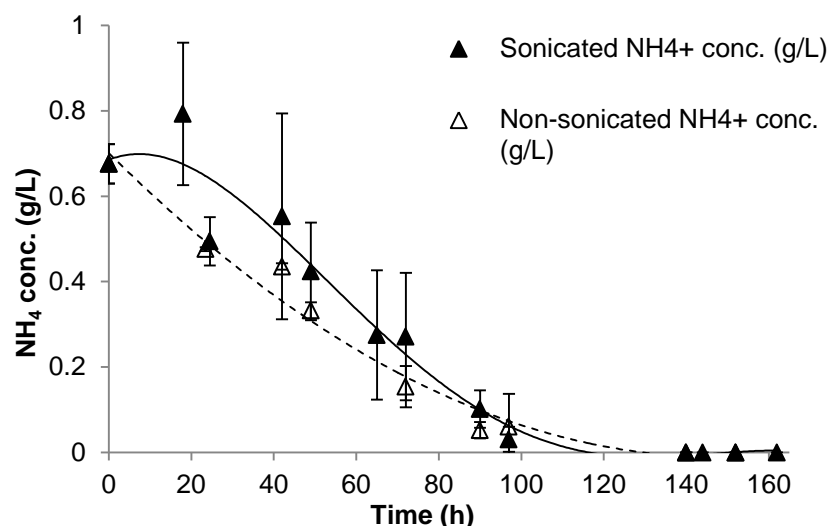


Figure 4.8: The ammonium concentration for the fermentation profile of *Rhodotorula glutinis* cultured with and without ultrasonication. 1.5 L of RSM medium was supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ at 28 °C with cascading agitation (set point, 25%) for 160 h. Sonication commenced at 42 h. The pH was uncontrolled throughout in all growth conditions. The error bars indicate the standard deviation from the mean where $n=3$. The lines represent a polynomial fit using Microsoft Excel.

4.3.2.2 Effect of sonication on the biomass and lipid productivity

For this sonication regime, the specific growth rate for the sonicated culture was 0.027 h^{-1} compared to 0.088 h^{-1} for the control (Table 4.3), indicating that the sonication regime was having a negative effect on cell growth. This was also observed with an intermittent sonication cycle commencing in the early exponential phase of *S. cerevisiae* growth. A 40% duty cycle (2 s sonication, 5 s rest period) was shown to be detrimental to growth, whereas shorter cycles increased biomass yields.²⁰⁰ This was also reflected in the final lipid yields of 9.30 % d.wt in the sonicated culture compared to 11.47 % d.wt in the control fermentation. However, the reduced uptake of glucose in the sonicated culture resulted in a comparable lipid yield (g of lipid per kg of glucose uptaken) between the fermentations of 37 g/kg and 50 g/kg for the sonicated and control cultures, respectively (Table 4.3). As the products of glucose metabolism are used for biosynthesis pathways other than purely in lipid production, even under ideal conditions for lipid biosynthesis, the lipid yield on glucose is very rarely more than 220 g/kg.^{22,35}

The lipid profile did not vary between the sonicated culture and the control culture, suggesting that the ultrasonication was not causing any adverse reactions such as isomerisation of the lipid. Compared to the lipid profiles observed in Chapter 3 for the same growth medium, the levels of oleic acid (18:1) were comparable to those yielded in the saturated *R. glutinis* oil, but the level of linolenic acid (18:2) was considerably higher at 27% (w/w) than ~10% (w/w) observed for *R. glutinis*. This type of lipid profile does not compare to that observed in terrestrial plants (Table 1.1), offering the potential for niche, high-value applications.

Table 4.3: Final biomass and lipid content of *Rhodotorula glutinis* when sonication was commenced at 42 h.

	Control culture	Sonication at 42 h
Maximum specific growth rate, μ (h^{-1})	0.088	0.027
Final Biomass (g L^{-1})	13.7	8.42 (± 2.09)
Biomass yield on glucose ($\text{g}_{\text{biomass}}/\text{kg}_{\text{glucose}}$)	490	390
Lipid (% dry weight)	11.47	9.30 (± 0.02)
Lipid co-efficient ($\text{g}/\text{kg}_{\text{glucose}}$)	50	37
Lipid profile (wt %)		
Palmitic acid (16:0)	27.3	24.5
Stearic acid (18:0)	0.8	0.6
Oleic acid (18:1)	45.7	45.9
Linoleic acid (18:2)	26.2	28.9

4.3.3 Two-stage fermentation process: *R. glutinis*

As the sonication had a negative effect on the growth of the yeast when applied in the exponential phase, the sonication was started at a later point in the cycle when the yeast had already achieved a high biomass concentration. At this point lipid accumulation can occur, and as such additional glucose was added to the broth to reduce the likelihood of sugar exhaustion that would halt lipid accumulation.

Similarly to the previous runs, the DO and pH reduced to 25% and 2.5, respectively, as the yeast entered the exponential phase (Figure 4.9). The DO of the control run started to increase after 70 hours, suggesting that the yeast had entered the decline growth phase. Similarly to the previous fermentation a spike was observed in the DO when the sonication was started. Interestingly, the DO remained reasonably stable from this point, roughly similar to the control, and both cultures

were still using oxygen by the end of the runs, suggesting that the sonication regime was not affecting the yeast metabolism severely.

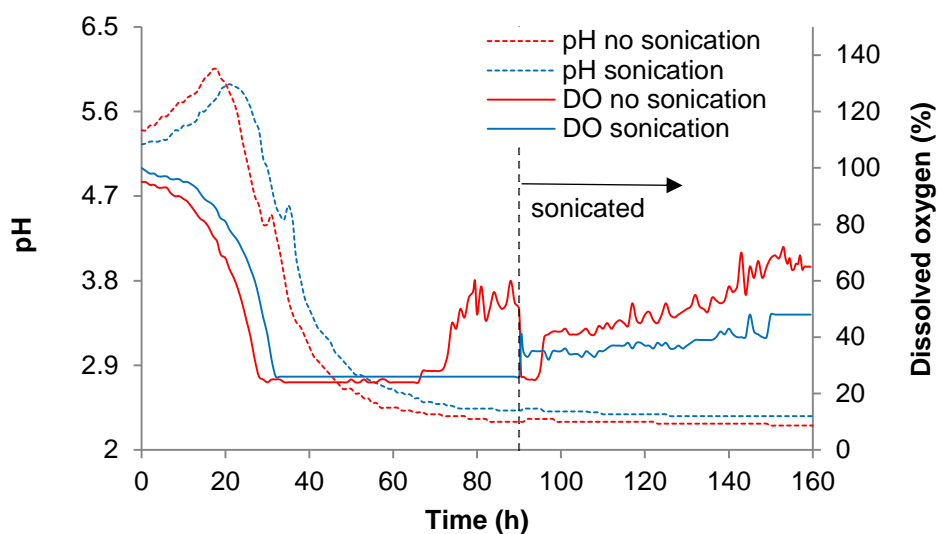


Figure 4.9: The pH and dissolved oxygen concentration for the fermentation profile for *Rhodotorula glutinis* for the sonicated (90 h) and non-sonicated cultures. 1.5 L of RSM medium was supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ and cultured at 28 °C with cascading agitation (set point, 25%) for 160 h. Sonication commenced at 90 h with the simultaneous addition of glucose (45 g). The pH was monitored but uncontrolled throughout. The data presented represents that obtained from one study under each condition, and was measured automatically, every 15 min, using biocommand Track and Trend software.

At 90 hours further glucose was added to the cultures (Figure 4.10). This two-step fermentation method is a common technique used to increase the C/N ratio of the medium and thus boost lipid productivity in single cell oils.⁷⁶ By 90 h there was also less than 0.1 g/L of nitrogen remaining in the culture medium (Figure 4.11). For the control run, the addition of glucose resulted in a large drop in the DO from 60% to 25% (Figure 4.28). This was presumably because, while lipid formation is essentially a series of reduction steps, requiring no oxygen to be synthesised from malonyl-CoA units, ATP and thus oxygen is still needed to produce malonyl-CoA from acetyl-CoA.²¹⁸ However, once the TCA cycle was saturated with its constituent intermediates, the DO then proceeded to increase. Following the addition of extra glucose, the control fermentation utilised glucose for a further 7 hours, with very little uptake after 97 h.

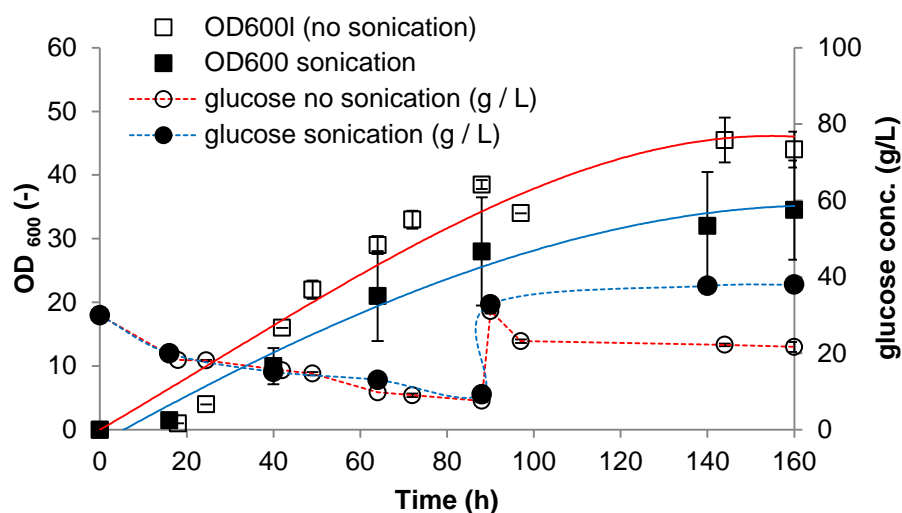


Figure 4.10: The optical density and glucose concentration for the fermentation profile for *Rhodotorula glutinis* cultured for the sonicated (90 h) and non-sonicated cultures. 1.5 L of RSM medium was supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ and cultured at 28 °C with cascading agitation (set point, 25%) for 160 h. Sonication commenced at 90 h with the simultaneous addition of glucose (45 g). The pH was uncontrolled throughout. The error bars indicate the standard deviation from the mean where $n=2$. The lines represent a polynomial fit using Microsoft Excel.

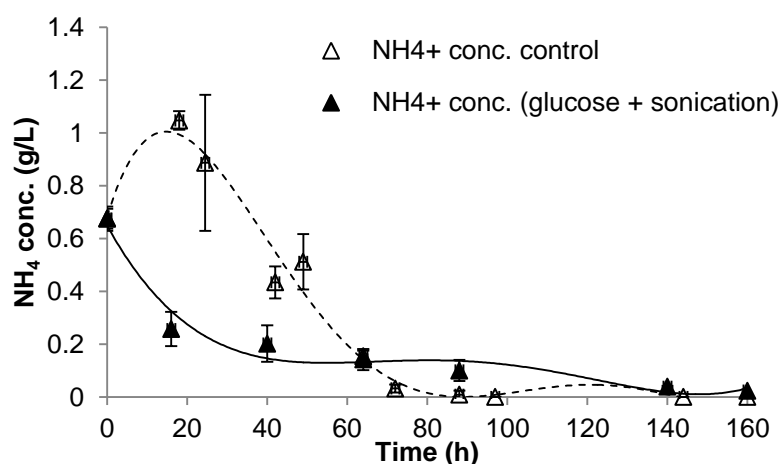


Figure 4.11: The ammonium concentration for the fermentation profile for *Rhodotorula glutinis* cultured in sonicated (90 h) and non-sonicated conditions. 1.5 L of RSM medium was supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$, at 28 °C with cascading agitation (set point, 25%) for 160 h. Sonication commenced at 90 h with the simultaneous addition of glucose (45 g). The pH was uncontrolled throughout. The error bars indicate the standard deviation from the mean where $n=3$. The lines represent a polynomial fit using Microsoft Excel.

4.3.3.1 Biomass and lipid productivity for the two-stage fermentation

Glucose consumption was comparable to the control prior to sonication, resulting in similar specific growth rates of 0.079 h^{-1} for the sonicated culture and 0.069 h^{-1} for the control fermentation during the exponential phase of cell growth (Table 4.4). No drop in the glucose content was observed for the sonicated *R. glutinis* fermentation following the initiation of sonication along with the addition of glucose. This resulted in a slightly greater final biomass yield of 12.66 g/L for the control culture compared to 11.71 g/L (560 g/kg) for the sonicated fermentation, although enhanced conversion of glucose to biomass was observed with sonication, ($460 \text{ g}_{\text{biomass}}/\text{kg}_{\text{glucose}}$ no sonication; $560 \text{ g}_{\text{biomass}}/\text{kg}_{\text{glucose}}$, sonication). Sonication also produced higher levels of oil per cell than when it was not used. The lipid production as a percentage of the dry cell weight was slightly higher compared to the control culture, resulting in a lipid yield of $130 \text{ g}_{\text{lipid}}/\text{kg}_{\text{glucose}}$. This was 3.5-fold greater than when the sonication regime commenced in early exponential phase. This indicates that more glucose was being converted into lipid rather than biomass, which is an essential consideration when the cost of the feedstock can be up to 70% of the final cost of the product.

Table 4.4: Final biomass and lipid content of *Rhodotorula glutinis* when sonication was commenced at 90 h, with a two-stage fermentation process.

	Control culture (+ glucose at 90 h)	Sonation and glucose at 90 h
Maximum specific growth rate, $\mu \text{ (h}^{-1}\text{)}$	0.069	0.079
Final Biomass (g L^{-1})	$12.66 (\pm 0.90)$	11.71
Biomass yield on glucose ($\text{g}_{\text{biomass}}/\text{kg}_{\text{glucose}}$)	460	570
Lipid (% dry weight)	$21.37 (\pm 0.06)$	22.27
Lipid co-efficient ($\text{g}/\text{kg}_{\text{glucose}}$)	100	130
Lipid profile (wt %)		
Palmitic acid (16:0)	25.4	25.6
Stearic acid (18:0)	0	0.9
Oleic acid (18:1)	47.0	46.0
Linoleic acid (18:2)	27.7	27.5

While this result is presumably more attributable to the increase in C/N ratio rather than a change to the sonication regime, sonication is known to aid the mass transfer of gases, and so can increase the level of oxygen in the cell, aiding in growth. There is however a balance between DO and lipid production. Low DO

retarded cell growth but increased lipid production in *R. glutinis* whereas high DO increased cell growth but decreased lipid accumulation. Similarly, lipid accumulation in *Candida (Yarrowia) lipolytica* was favoured at low dissolved oxygen levels.²⁰⁵ This is also true for the anaerobic production of ethanol from *S. cerevisiae*, but was enhanced with certain sonication regimes.²⁰⁰ As carbon dioxide is known to inhibit *S. cerevisiae*,²¹⁹ it is thought that the improved mass transfer may contribute to removal of the inhibitory CO₂ from the broth. Another key factor is that lipid accumulation is a stress response, be it to nitrogen-, sulphate-⁸⁷ or phosphate-limited¹⁶⁶ medium or to changes in temperature.¹⁷⁴ It is possible that by putting the yeast under further stress caused by the sonication regime, lipid accumulation is enhanced.

4.3.4 Two-stage fermentation process: *R. minuta*

As it was only possible to run the sonication experiments on one fermenter, in order to make use of the fermentation equipment available at Almac, *R. minuta* was cultured in a two-stage process without the inclusion of sonication. *R. minuta* was cultivated in the same RSM medium used previously, with the addition of 45 g glucose at 97 h when the yeast had exhausted the nitrogen available in the culture medium (Figure 4.13). The growth profile exhibited by *R. minuta* was very similar to that observed by *R. glutinis*, in which an initial drop in pH was coupled with an increase followed by a substantial decrease to pH 2.5 (Figure 4.12). The DO level strongly mirrored this pattern, with lower DO levels observed at lower pH levels. As DO is strongly linked to the growth of microorganisms, this suggests that elevated pH are not conducive to the growth of *R. minuta*. From approximately 40 h, the DO level within the culture medium began to increase which suggested that it entered the decline phase of growth much earlier than *R. glutinis*. A substantial amount of growth was however seen after this point (Figure 4.13). A similar drop in the DO on the addition of extra glucose was also observed for *R. minuta* as described previously for *R. glutinis*.

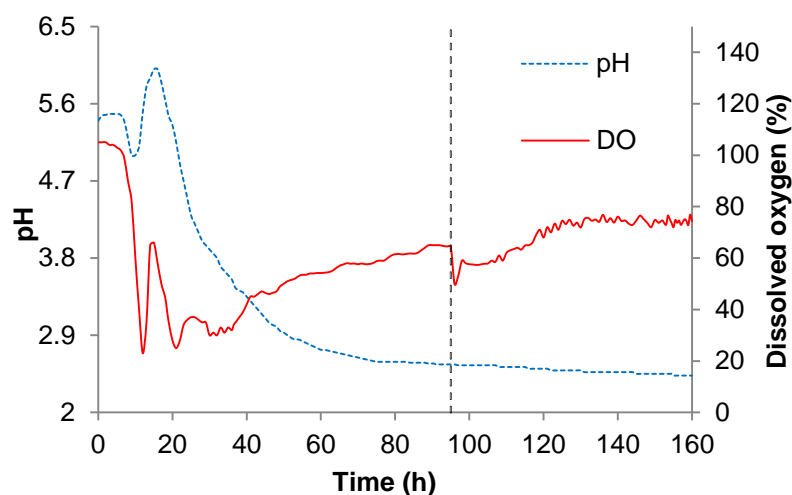


Figure 4.12: The dissolved oxygen concentration (DO) and pH for the fermentation profile of *Rhodotorula minuta* grown under a two-stage lipid accumulating regime. 1.5 L of RSM medium (30 g/L glucose, 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$), with uncontrolled pH and with cascading agitation (set point, 25%) for 160 h. Additional glucose (45 g) was added at 97 h, represented by the vertical dashed line. The data represents a one-off scoping study. pH and DO were measured automatically, every 15 min, using Biocommand Track and Trend software.

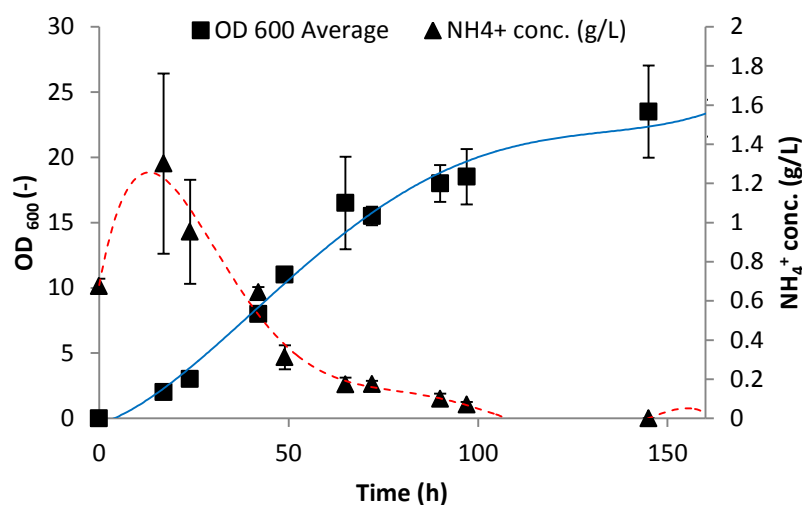


Figure 4.13: The ammonium concentration and optical density at 600 nm (OD_{600}) for the fermentation profile of *Rhodotorula minuta* grown under a two-stage lipid-accumulating regime. 1.5 L of RSM medium (30 g/L glucose, 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$), with uncontrolled pH and cascading agitation (set point, 25%) for 160 h. Additional glucose (45 g) was added at 97 h. The error bars indicate the standard deviation from the mean where $n=2$. The lines represent a polynomial fit using Microsoft Excel.

When comparing the productivity of the system from *R. minuta* (Table 4.5) to that of *R. glutinis* (Table 4.4), the former system was not as productive. While the lipid content of the cell was similar between the two species, at 21% (d.wt), the biomass

produced was much lower for *R. minuta* than for *R. glutinis*, at 6.96 g/L and 12.66 g/L, respectively. The biomass and lipid coefficients for *R. minuta* were therefore lower at 385 g/kg and 83 g/kg, respectively, compared to 460 g/kg and 100 g/kg, for *R. glutinis*. Although, the biomass and lipid coefficients produced from *R. minuta* in this study are low, they are similar to those obtained from the shake flask experiments in Chapter 3 (Table 3.4), suggesting that *R. minuta* growth can be representatively scaled to small fermenters.

Table 4.5: Final biomass and lipid content of *Rhodotorula minuta* when sonication was commenced at 90 h, with a two-stage fermentation process.

	<i>R. minuta</i>
Maximum specific growth rate, μ (h^{-1})	0.034
Final Biomass (g L^{-1})	6.96 (± 0.18)
Biomass yield on glucose ($\text{g}_{\text{biomass}}/\text{kg}_{\text{glucose}}$)	385
Lipid (% dry weight)	21.56 (± 2.09)
Lipid co-efficient ($\text{g}/\text{kg}_{\text{glucose}}$)	83
Lipid profile (wt %)	
Palmitic acid (16:0)	23.7
Stearic acid (18:0)	4.6
Oleic acid (18:1)	41.1
Linoleic acid (18:2)	30.7
Linolenic acid (18:3)	0

4.4 Conclusion

pH control negatively affected both biomass production and lipid accumulation in *R. glutinis*. While the affect appears to be strain-specific, the same growth pattern has been observed previously for *R. glutinis*, especially when ammonium salts were used as the nitrogen source.²²⁰ The ultrasound regime was found to have no effect on the lipid profile of the yeast, despite *R. glutinis* being highly sensitive to environmental changes.²²¹ The application of ultrasound was found to have no positive effect on the biomass growth or lipid accumulation when applied in the exponential phase. However, on applying sonication in the stationary phase, beneficial impacts were observed with the lipid coefficient being increased by 30% from 100 g / $\text{kg}_{\text{glucose}}$ to 130 g / $\text{kg}_{\text{glucose}}$. Despite the increase in cellular lipid production, the overall system did not create more lipid than the control (with no ultrasound), due to the reduced biomass production of *R. glutinis*, though increased biomass and lipid production per unit input of glucose was observed with

ultrasound. The results observed under the sonication regime are however unlikely to be substantial enough to justify the cost surrounding the experimental set-up. Growth of *R. minuta* within a 2.5 L fermenter was demonstrated, and while the productivity of the system in terms of biomass and lipid production were less than the equivalent study for *R. glutinis*, the lipid and biomass coefficients obtained were similar to those previously presented from shake flask experiments. While this chapter has successfully demonstrated the growth of *R. glutinis* within industrial fermenters, another important consideration is the production of single cell oil from a cheap, renewable feedstock rather than highly refined glucose.

CHAPTER 5:

THE TRANSITION TO WASTE RESOURCES

“There is fuel in every bit of vegetable matter that can be fermented.”

Henry Ford

5.1 Preamble

While high oil productivity, fast growth rates and a suitable fatty acid composition are key traits for the economic viability of SCO production, it is essential that the organism can be cultured and produce lipids from low-cost substrates.²¹ This requires the ability to assimilate the nutrients present in the desired hydrolysate, as well as maintain high growth rates in the presence of inhibitory compounds produced during the hydrolysis process. Work presented in this chapter details the transition to growing *Rhodotorula sp.* on waste resources, with a particular focus on food waste hydrolysate.

5.2 Introduction

There is growing interest on the use and valorisation of biomass feedstocks for the production of bioenergy and as a sustainable source for commodity products such as chemicals, oil and plastics.²²² *Second generation* feedstocks, that do not compete with the food supply chain include energy crops, wood, microbial biomass as well as wastes from household, agricultural, cattle, forestry and industrial processes.²²³ One of the most abundant biomass sources is lignocellulose, resulting from agricultural residues such as corn stover, wheat straw and bagasse (sugar cane),²²⁴ waste food or fast-growing energy crops such as switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus giganteus*) and giant reed (*Arundo donax*).⁸⁵

5.2.1 Lignocellulose as a sugar source

Lignocellulose is composed of up to 75% carbohydrates and thus offers a valuable source of fermentable sugars.²²⁵ It is a complex matrix of carbohydrate polymers, composed primarily of cellulose (~45%), hemicellulose (~30%) and lignin (~15%). The relative abundance of these components depends on the plant species, age and growth conditions.²²⁵ The extraction of the sugars, mainly from the cellulose fraction is however one of the key challenges to biorefineries. Cellulose is the most abundant constituent of the plant biomass, and it consists entirely of glucose molecules linked together by β -1,4-glycosidic bonds. These glucose chains can be up to 10,000 molecules in length, and cluster together to form cellulose fibrils with a highly crystalline supramolecular structure. Hemicellulose is a much more

complex polysaccharide, containing several sugar molecules including D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose and D-glucuronic acid. The relative distribution of these sugars within the hemicellulose is species-specific. For example, grasses such as switchgrass or wheat straw contain mostly glucuronic acid, arabinose and xylose whereas softwoods such as spruce or pine contain galactose, glucuronic acid and xylose. Hemicellulose polysaccharides are generally shorter in length (<200 molecules) than those contained within cellulose, and they may have side chains or be acetylated. Within a plant cell wall, the hemicellulose surrounds the cellulose fibrils, which can protect the cellulose from enzymatic hydrolysis. The whole structure is enveloped by lignin; a highly complex aromatic polymer, which provides much of the mechanical strength to the plant cell wall. It constitutes 15-40% of the biomass dry weight, and in polysaccharide processing, it is generally removed from the plant material unhydrolysed and burned to provide the energy for the processing plant. Research is however underway to make use of this chemically complex polymer for value-added products.²²⁶

5.2.2 Municipal organic waste

The effective management of municipal waste is of increasing importance globally, and over the past decade the waste landscape has changed drastically. The EU Landfill and Waste Framework directive developed specific targets for product and energy recovery as well as diverting waste to landfill.²²⁷ By 2016, the amount of biodegradable municipal waste permitted in landfills will be capped at 420,000 tonnes/annum. Food waste makes up the largest proportion of the organic fraction within municipal waste, and if managed incorrectly, it can contaminate recyclable material or produce methane gas if disposed of in landfill.²²⁸ It is estimated that 30-50% of the food produced globally, equivalent to 1-2 billion tonnes, is not used for human consumption.²²⁹ In the EU in 2006, household food waste was estimated to be 37.7 million tonnes, representing 42% of all food wastes generated in the EU.²³⁰ Therefore, household food waste poses a disposal challenge as well as providing an attractive feedstock for further valorisation. Several EU states have acknowledged that in order to divert food waste away from landfill, effective source separation is required. As of 2013, household food waste segregation in the Republic of Ireland

was made obligatory following the introduction of new regulations to meet the objectives outlined in the landfill directive. In the UK, several councils have been trialling specific food waste bins to reduce the amount of food waste disposed of into landfill.²³¹ Developing an effective recycling and valorisation system is therefore extremely attractive.^{222, 232}

5.2.2.1 Biotransformation of food waste

Due to the high water content of food waste, the biological transformation of food waste is energetically more favourable than combustion or gasification.²³³ The inherent chemical complexity of food waste also makes it an attractive feedstock for higher value products. While the composition varies depending on the feedstock source, food waste generally contains carbohydrate polymers (starch, cellulose, hemicellulose), lignin, proteins, fats, organic acids and a smaller (~5%) inorganic/ash fraction.⁸³ It has been used as the sole microbial feedstock for the development of various value-added products such as methane, hydrogen, ethanol, enzymes, organic acids, biopolymers and bioplastics.⁸³ For example, Hong *et al.* achieved yields of 60 g reducing sugar /100 g food waste, resulting in 0.36 g ethanol /g food waste using *S. cerevisiae*.²³⁴ More recently, Chi *et al.* cultivated *C. curvatus*, *Y. lipolytica*, *L. starkeyi*, *R. toruloides* and *R. glutinis* on hydrolysed food waste for SCO production. While the growth of *L. starkeyi* and *R. toruloides* were considerably reduced, *C. curvatus*, *R. glutinis* and *Y. lipolytica* produced biomass yields equal to or better than the control when cultivated on food waste hydrolysate. Only *R. glutinis* and *C. curvatus* were tested for SCO production, producing lipid concentrations (%dwt) of 19.6% and 28.6%, respectively, thus demonstrating the ability to produce SCO from food waste.⁸⁴

5.2.3 Hydrolysis of biomass feedstocks

Because yeast are incapable of significant cellulolytic activity, the lignocellulose or municipal organic waste must be pretreated and hydrolysed to release the sugars prior to conversion by oleaginous yeast (also referred to as depolymerisation or saccharification). The cost of the feedstock can be up to 70% of the final production cost, and as such it is essential to enhance sugar yields from the biomass feedstock because of its impact on the downstream processing costs. The pretreatment step

can contribute considerable costs to the whole process, but as Wyman stated, *“the only step more expensive than pretreatment is no pretreatment”*.²³⁵ Various depolymerisation methods have been used to extract the soluble sugars from lignocellulosic biomass, with the conventional method involving dilute acid pretreatment followed by enzymatic hydrolysis.⁸⁵ Aqueous ammonia soaking of lignocellulosic biomass can also be an effective method to increase the sugar yield from the biomass, as it has high selectivity towards lignin and as such does not disrupt the carbohydrate structure. It can also cause swelling of the cellulose fibrils, and thus aids later depolymerisation techniques.²³⁶ Regarding food waste, various depolymerisation methods have been employed previously including: enzymatic,²³² thermo-chemical,^{84,237} combined thermo-chemical and enzymatic,²³⁸ and whole-cell fungal hydrolysis.²³⁹

Most commonly, the hydrolysis and fermentation steps occur separately, but with an aim to improve the economics of the system, simultaneous saccharification and fermentation (SSF) is becoming increasingly common.²⁴⁰⁻²⁴¹ When using enzymes, this also reduces the effects of product inhibition of the enzymes which decreases the hydrolysis efficiency.²⁴² More recently, consolidated bioprocessing (CBP) has also received considerable attention, in which the fermenting organism also releases the cellulases (enzymes) necessary for the saccharification of the biomass.²⁴³

5.2.3.1 Sugar catabolism

Depolymerised biomass feedstocks mainly contain glucose and xylose, generally in a 2:1 ratio,¹⁹⁰ although the relative proportion of these depends on the method and source of the feedstock.²⁴⁴ Other monomeric sugars include arabinose, glucuronic acid, and mannose, and the oligosaccharides cellobiose, cellotriose, maltose and maltotriose. In waste streams, such as food waste, the disaccharides lactose, sucrose and fructose are also common. To increase the efficiency of the overall process it is vital that the oleaginous organism can metabolise all of the sugars available. While many carbon sources have been considered as substrates for the production of lipid from oleaginous yeast, including simple sugars such as glucose, fructose and glycerol, several organisms cannot naturally assimilate C₅ sugars such

as xylose. In order to utilise the full range of sugars available, xylose-based media have attracted considerable interest.⁸⁵ For example, the ethanol-producing yeast, *Saccharomyces cerevisiae*, the yeast most widely used in industrial biotechnology, is unable to ferment xylose without genetic modification. Bettiga *et al.* inserted a xylose utilisation pathway resulting in xylose uptake and ethanol production,²⁴⁵ but even the strains that have been engineered to ferment xylose generally do so slowly, and cannot utilise xylose until glucose is completely consumed.²⁴⁶ Genetically modified *S. cerevisiae* have also showed lower ethanol yields and reduced tolerance to ethanol compared to the optimised glucose fermentation, further hindering their utilisation.²⁴⁷ It therefore seems that a more promising route is to use alternative yeast that are naturally able to assimilate carbon from multi-sugar feedstocks.

Even for organisms that can assimilate both C₅ and C₆ sugars, when both glucose and xylose are present in the growth medium a pattern of diauxic growth can arise in which one substrate is catabolised preferentially over the others, with a lag period occurring between the growth phases.²⁴⁸ Generally, glucose is utilised during the first growth phase as it can allosterically repress other sugar transporters.¹⁹⁰ The delay between growth phases is also due to the synthesis of the enzymes necessary for xylose metabolism.²⁴⁸ This pattern of sequential glucose and xylose uptake was observed in *Lipomyces starkeyi*¹⁷⁹ and *Rhodotorula glutinis*.²⁴⁹ In contrast, the oleaginous yeast, *Trichosporon cutaneum* was able to assimilate glucose and xylose simultaneously, with no pattern of diauxic growth observed.¹⁹⁰

5.2.3.2 Inhibitory compounds

Under the depolymerisation conditions, the sugars as well as residual lignin, can degrade further to yield a range of toxic compounds that are inhibitory to microbial growth and therefore lipid productivity. Xylose can be degraded into furfural, and 5-hydroxymethylfurfural (5-HMF) is produced from glucose.²⁴² When these compounds are further degraded, formic acid and levulinic acid are produced from furfural and 5-HMF, respectively. The breakdown of lignin produces phenolic compounds such as vanillic acid, vanillin, syringic acid, syringaldehyde and 4-hydroxybenzoic acid (Figure 5.1).^{21,242} The relative concentrations of these

compounds depends on the feedstock used as well as the depolymerisation method.²⁵⁰ These compounds do, however, have the potential to be removed as high-value chemical intermediates.²¹

The inhibitors can be removed from the hydrolysate through overliming or with activated charcoal,²⁵¹ though this would increase the costs of the processing substantially when compared to using a yeast strain with natural inhibitor tolerance. Chen *et al.* tested the effects of some common inhibitors on the growth and lipid productivity of five yeast species, including three strains of *R. glutinis* and concluded that the effects were inhibitor and strain specific.²⁵⁰ Weak acids, such as acetic-, levulinic- and formic- acid are believed to hinder cell growth due to the ability of the undissociated acid being able to diffuse across the cell membrane, thus disrupting the neutral pH of the cytosol. A linear relationship between the cell replicative activity and decreasing intracellular pH has been previously observed.²⁴² Generally, Ascomycetes yeast can tolerate acetic acid better than Basidiomycetes yeast.²⁵² This was believed to be due to the acetic acid repressing Basidiomycetae growth, rather than the ability to grow at a lower pH. Furfural is known to be a strong inhibitor of yeast growth,²⁵² with many yeast species unable to tolerate concentrations higher than 1 g/L, and delayed growth exhibited at 0.5 g/L.²⁵² 5-HMF is less toxic to yeast than furfural, but many oleaginous yeast can only tolerate concentrations up to 2 g/L,²⁵⁰ and exhibit delayed growth.²⁵²

5.2.4 Aims of the chapter

This chapter aims to investigate the growth of *Rhodotorula* sp. when a variety of compounds that result from the depolymerisation of biomass feedstocks are present. Firstly, microbial growth kinetics on xylose and glucose mixtures will be tested, before assessing the yeast growth on a selection of mono- and di-glycerides. Tolerance of *Rhodotorula* sp. to a range of common microbial inhibitors will also be tested. Finally, yeast growth on depolymerised *Miscanthus* and food waste will be performed to assess the feasibility of SCO production under real-life scenarios.

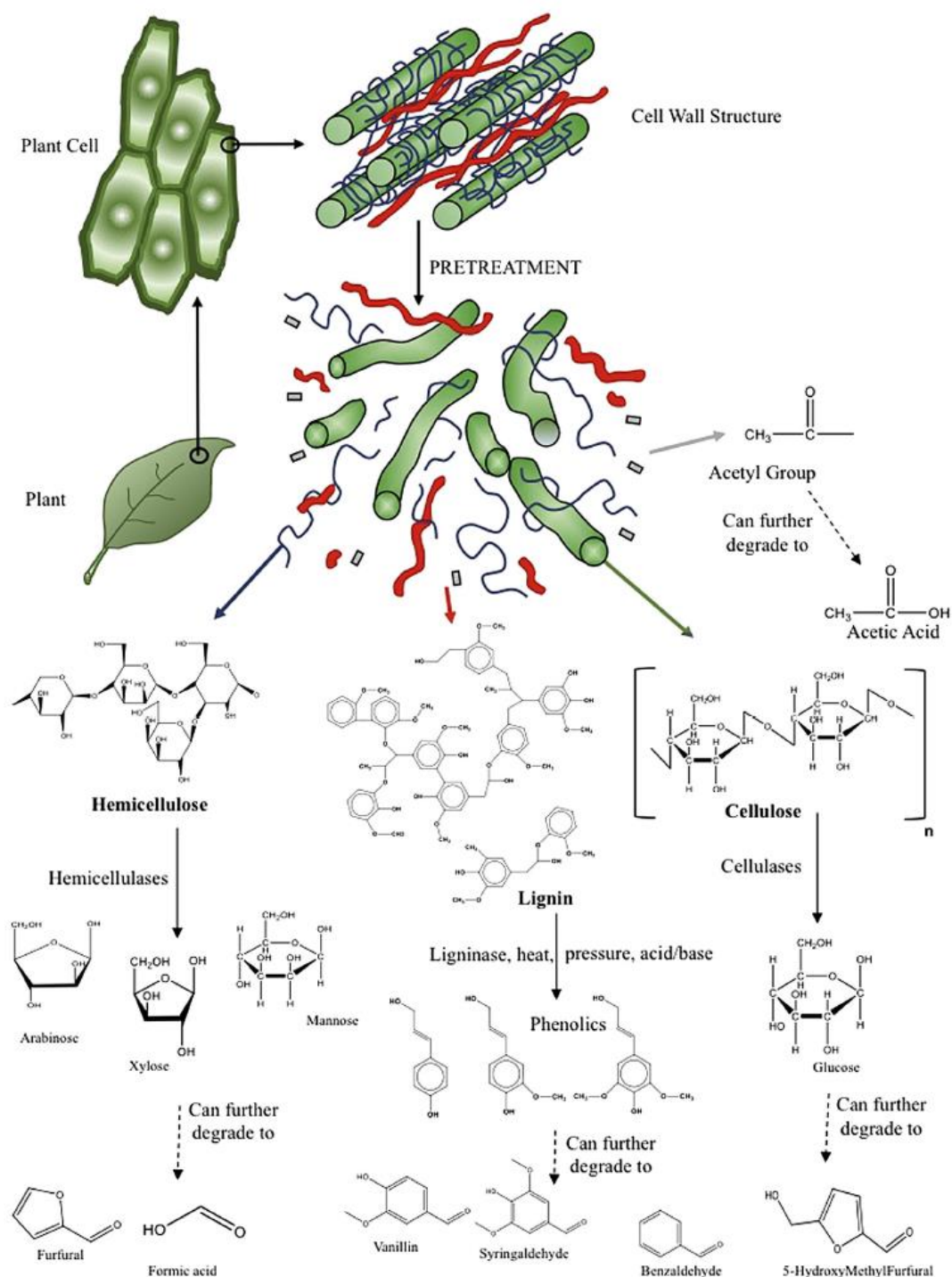


Figure 5.1: Lignocellulose break-down products resulting from the pre-treatment and hydrolysis of biomass. The common sugars resulting from cellulose and hemicellulose degradation, and the resulting inhibitors, as well as the phenolic compounds from lignin degradation are depicted.²¹

5.3 Results and discussion

5.3.1 Transition to renewable feedstocks

In order for the economics of microbial lipid production from lignocellulose to succeed, the oleaginous microbe must have the ability to metabolise C₅ (e.g. xylose) as well as C₆ sugars (e.g. glucose). For an industrial process, it would be beneficial to employ a yeast which can naturally metabolise glucose and xylose simultaneously, without evidence of a diauxic growth pattern. Dai *et al.* previously demonstrated that *R. glutinis* is capable of assimilating xylose,²⁵³ although the growth kinetics over a range of xylose concentrations were not compared. Less is known surrounding the growth characteristics of *R. minuta*. Therefore, *R. glutinis* and *R. minuta* were cultured with varying levels of glucose and xylose over 120 hours, and the sugar uptake kinetics examined.

5.3.1.1 *R. glutinis* growth on C₅ and C₆ sugars

R. glutinis was cultured under different concentrations of glucose and xylose in a growth medium containing a total sugar concentration of 30 g/L, ranging from 100% glucose (wt.%) to 100% xylose (wt.%), (Table 5.1).

Table 5.1: Growth kinetics of *Rhodotorula glutinis* on combinations of glucose and xylose.

Glucose (wt.%) [†]	Xylose (wt.%) [†]	Rate constant of glucose metabolism (h ⁻¹)	R ²	Rate constant of xylose metabolism (h ⁻¹)	R ²	OD ₆₀₀ at 120 h (-)
100	0	6.7 x 10 ⁻³	0.99	-	-	15.02 ± 3.50
75	25	5.9 x 10 ⁻³	0.99	6.2 x 10 ⁻³	0.96	14.34 ± 3.19
50	50	6.1 x 10 ⁻³	0.98	4.3 x 10 ⁻³	0.95	13.51 ± 2.91
25	75	9.0 x 10 ⁻³	0.99	3.3 x 10 ⁻³	0.94	13.34 ± 2.99
0	100	1.4 x 10 ^{-2‡}	0.97	3.6 x 10 ⁻³	0.95	12.22 ± 2.50

[†]Weight percent of total sugar concentration. The combined sugar concentration was maintained at 30 g/L. [‡]Residual glucose present from the inoculum

Zhang *et al.* observed a first order relationship for the specific growth rate of *R. glutinis* when assessing cell concentration in relation to time.⁵⁷ A plot was therefore constructed with respect to glucose or xylose concentration for each of the varying sugar concentrations to determine the rate constant, *k*, (as described in Section 2.3.2.1) which was plotted against the biomass productivity for each growth culture (Figure 5.2). For each of the sugar concentrations, a first order linear regression was

obtained suggesting that sugar assimilation in *R. glutinis* does indeed follow first order kinetics. The correlation coefficients (R^2) obtained ranged from 0.94 – 0.99, again supporting the notion of a first order reaction. In reality, this is most likely to be pseudo-first order due to the high excess of glucose and xylose in many of the culture media.

The rate constant for glucose consumption was found to be $6.7 \times 10^{-3} \text{ h}^{-1}$ when no xylose was present. The rate of glucose uptake remained reasonably stable around $6.0 \times 10^{-3} \text{ h}^{-1}$ until only 25% of the sugar present in the growth medium was glucose. At this point the rate constant increased dramatically to $9.0 \times 10^{-3} \text{ h}^{-1}$. As the level of xylose in the culture medium increased, the rate of xylose metabolism decreased. Even when there was only xylose present in the growth medium, the rate constant was only $3.6 \times 10^{-3} \text{ h}^{-1}$, suggesting that for the maximum growth to be achieved, glucose needs to be present in the culture medium. In all the glucose:xylose mixtures, glucose and xylose were assimilated simultaneously, and thus no evidence of the classic diauxic behaviour was observed. However, as the glucose concentration decreased in the culture medium, so too did the final biomass concentrations. These decreased from an optical density of 15.02 (-) at 100% glucose, to 12.22 when the growth medium contained only xylose as the sugar substrate (Table 5.1).

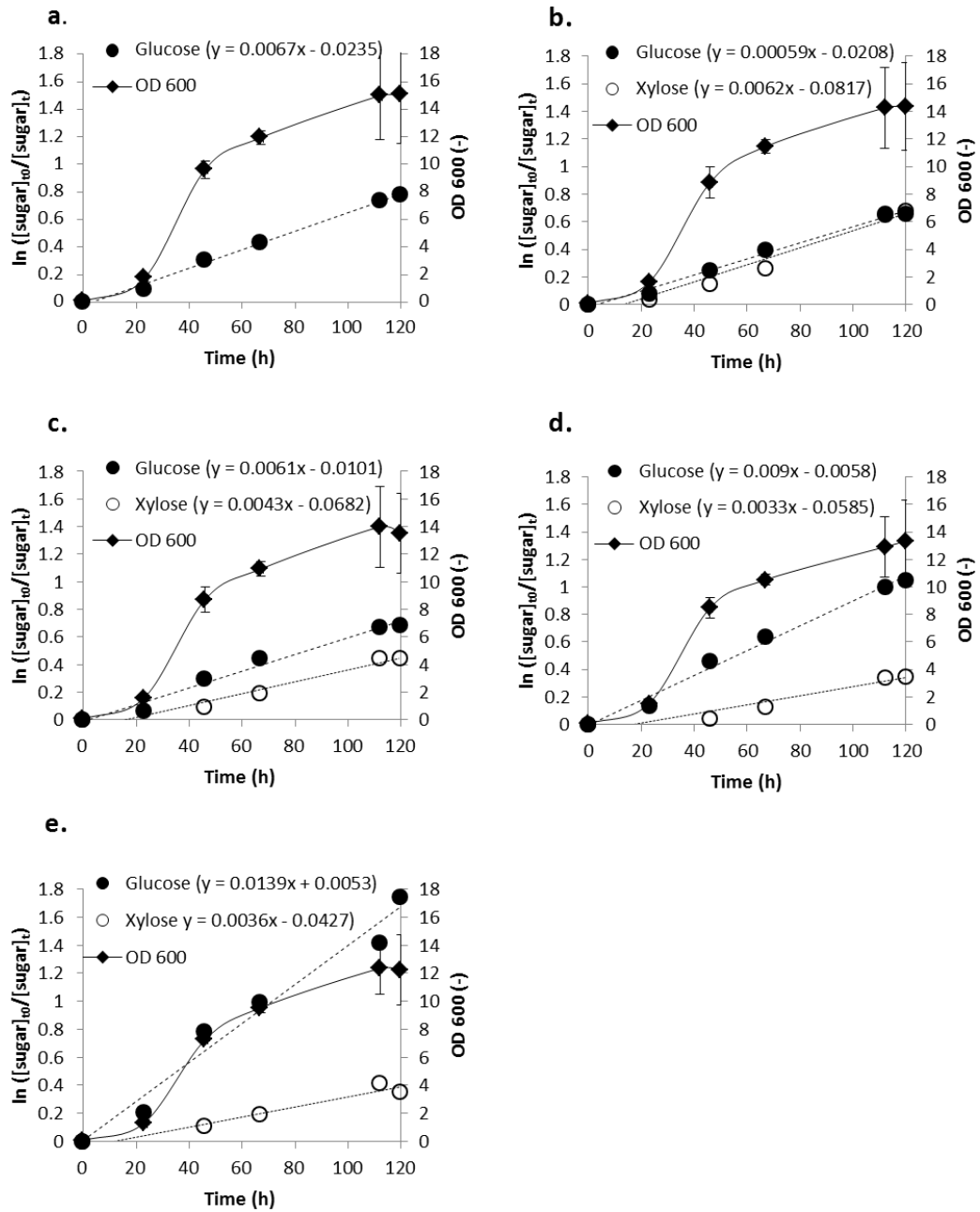


Figure 5.2: Sugar kinetics for *Rhodotorula glutinis*. a. 30 g/L glucose; b. 22.5 g/L glucose, 7.5 g/L xylose; c. 15 g/L glucose, 15 g/L xylose; d. 7.5 g/L glucose, 22.5 g/L xylose; e. 30 g/L xylose, cultivated in RSM medium supplemented with the appropriate quantity of sugar with 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ at 28 °C for 120 h, 180 rpm. The error bars represent the standard deviation from the mean where $n=3$. The square parentheses indicate the concentration of sugar. Filled and open circles represent the rate constant for glucose and xylose, respectively. Filled diamonds represent optical density at 600 nm.

5.3.1.2 *R. minuta* growth on C₅ and C₆ sugars

Similarly to *R. glutinis*, assuming a first order relationship, the rate constant for glucose consumption for *R. minuta* was found to be $6.5 \times 10^{-3} \text{ h}^{-1}$ when no xylose

was present. The pattern of glucose metabolism was similar for *R. minuta* as it was for *R. glutinis*, maintaining a relatively stable rate constant around $5.5 \times 10^{-3} \text{ h}^{-1}$, until the concentration of glucose decreased to only 25%, at which point the rate of glucose metabolism increased to $9.8 \times 10^{-3} \text{ h}^{-1}$ (Table 5.2). The xylose rate constant was relatively stable at around $3.0 \times 10^{-3} \text{ h}^{-1}$, regardless of the xylose concentration in the growth medium. As was observed for *R. glutinis*, the biomass, measured through optical density, decreased as the relative proportion of xylose in the culture medium increased. This decreased from 14.35 (-) at 100 wt.% glucose to 9.86 (-) when only xylose was present in the culture medium. No subsequent sugar uptake was observed for *R. minuta* although glucose does need to be present in the growth medium in order to obtain higher biomass yields (Figure 5.3).

Table 5.2: Growth kinetics of *Rhodotorula minuta* on combinations of glucose and xylose.

Glucose (wt.%) [†]	Xylose (wt.%) [†]	Rate constant of glucose metabolism (h^{-1})	R^2	Rate constant of xylose metabolism (h^{-1})	R^2	OD ₆₀₀ at 120 h (-)
100	0	6.5×10^{-3}	0.99	-	-	14.35 ± 3.04
75	25	5.0×10^{-3}	0.99	3.4×10^{-3}	0.94	12.84 ± 3.23
50	50	5.9×10^{-3}	0.99	2.6×10^{-3}	0.94	12.30 ± 2.43
25	75	9.8×10^{-3}	0.99	2.7×10^{-3}	0.98	11.85 ± 2.15
0	100	$1.3 \times 10^{-2}^{\ddagger}$	0.96	3.0×10^{-3}	0.98	9.86 ± 2.19

[†]Weight percent of total sugar concentration. The combined sugar concentration was maintained at 30 g/L. [‡]Residual glucose present from the inoculum.

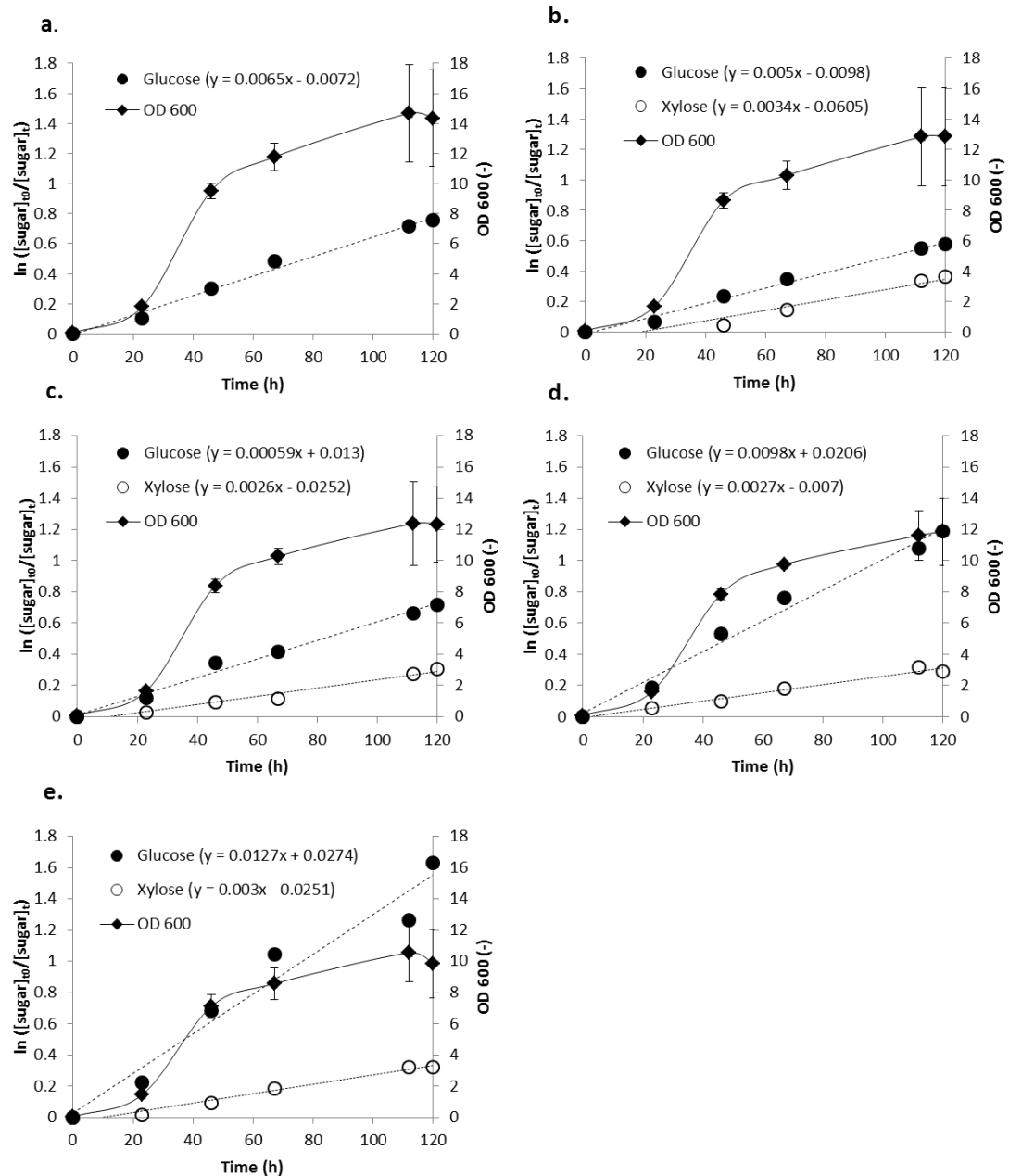


Figure 5.3: Sugar kinetics for *Rhodotorula minuta*. a. 30 g/L glucose; b. 22.5 g/L glucose, 7.5 g/L xylose; c. 15 g/L glucose, 15 g/L xylose; d. 7.5 g/L glucose, 22.5 g/L xylose; e. 30 g/L xylose. Cultivated in RSM medium supplemented with the appropriate quantity of sugar with 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ at 28 °C for 120 h, 180 rpm. The error bars represent the standard deviation from the mean where $n=3$. The square parentheses indicate the concentration of sugar. Filled and open circles represent the rate constant for glucose and xylose, respectively. Filled diamonds represent optical density at 600 nm.

5.3.1.3 Comparison of *Rhodotorula sp.* C₅ and C₆ sugar catabolism

For both *R. glutinis* and *R. minuta*, the glucose and xylose were assimilated simultaneously rather than sequentially. This is in contrast to Zhang *et al.* who reported no assimilation of xylose when both glucose and xylose were present in the culture medium during the growth of *R. glutinis*.⁵⁷ For both species tested in

this study, the rate of glucose metabolism increased as the concentration in the culture medium decreased. For *R. glutinis* the same effect was observed for xylose, although the rate of xylose uptake was slower than that of glucose. In contrast, the rate of xylose uptake remained stable around $3.0 \times 10^{-3} \text{ h}^{-1}$ for *R. minuta*, regardless of the xylose concentration. This is indicative of two different sugar uptake mechanisms. For *Candida shehatae*, a yeast with natural xylose assimilating capability, two kinetically distinct xylose-transport systems have been identified.²⁵⁴ One of the transport systems is shared with glucose by a facilitated diffusion pathway, whereas the other is a high-affinity xylose-proton symporter that also accepts glucose as a substrate.²⁵⁵ Glucose can however allosterically repress other sugar transporters, and as such it is generally metabolised first.¹⁹⁰

When glucose and xylose were present in equal concentrations, the rate of glucose metabolism was approximately double that of the xylose metabolism for *R. minuta* (Figure 5.3, c), whereas it was only ~50% higher for *R. glutinis* (Figure 5.2, c). Both yeasts showed a reduction in biomass productivity as the proportion of xylose within the culture medium increased. This pattern has also been reported previously during glucose and xylose fermentation by *Trichosporon cutaneum*, a yeast which can simultaneously utilise glucose and xylose but yielded lower biomass and lipid yields as the proportion of xylose within the culture medium increased.¹⁹⁰ Stoichiometrically, xylose metabolism is the more efficient route for lipid synthesis, yielding a maximum yield of $0.34 \text{ g}_{\text{lipid}}/\text{g}_{\text{xylose}}$ compared to $0.32 \text{ g}_{\text{lipid}}/\text{g}_{\text{glucose}}$.⁹⁶ Lipid production was also reduced compared to the growth on solely glucose when *R. toruloides* was grown exclusively on xylose as well as in a mixed-sugar medium containing glucose, xylose and arabinose. However, in the latter case the sugars were assimilated consecutively in the order described.²⁵⁶

5.3.2 Alternative sugar utilisation

One important industrial characteristic for oleaginous yeast is the utilisation of a broad range of carbon sources other than solely glucose and xylose. Sugars were selected that are present in various lignocellulosic hydrolysates and waste streams. Glucose, xylose, arabinose and cellobiose constitute a large portion of simple sugars present in depolymerised lignocellulose, whereas sucrose and fructose are more

likely to be present in hydrolysed food waste streams. Waste streams, such as waste whey is also rich in lactose.⁷⁰

Both *R. glutinis* and *R. minuta* were tested for their ability to assimilate a range of alternative sugar sources, as the sole carbon source (30 g/L) or in a 50:50 (w/w) blend ratio with glucose or xylose (Figure 5.4). The growth obtained from each of the conditions was compared to that of glucose, set at 100%.

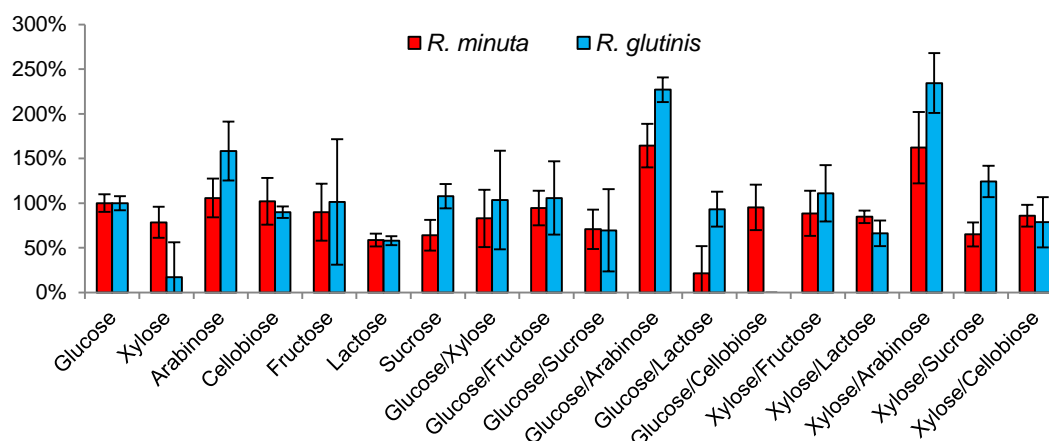


Figure 5.4: Biomass productivity for *Rhodotorula minuta* and *Rhodotorula glutinis* on a range of simple sugars. OD₆₀₀ measurement was taken after 5 days. The OD₆₀₀ has been standardised to a percentage of the growth on glucose. The yeast were cultured a range of simple sugars (30 g/L per culture) in 96 well plates at 28 °C. The error bars show the standard deviation from the mean where n=6.

Similar biomass productivities were observed for *R. minuta* grown on arabinose and fructose as that grown on glucose. Slightly less biomass was produced from growth on xylose which is similar to the results presented previously. While *R. minuta* grew extremely well on the disaccharide cellobiose, it was less effective on lactose and sucrose, resulting in less than 50% of the biomass observed when grown on glucose. In mixed sugar cultures, *R. minuta* grew extremely well on mixtures containing arabinose, producing over double the amount of biomass compared to *R. minuta* growth on glucose. These results are comparable to Sitepu *et al.* who demonstrated the growth of *R. minuta* on glucose, xylose and arabinose as well as less growth when sucrose was used as the carbon source.²⁵² Their results did however show less growth on cellobiose, in contrast to the strain tested here.

R. glutinis grew particularly well on substrates containing arabinose, producing over double the biomass of cultures with glucose alone when mixed with either glucose or xylose. In contrast, *R. glutinis* showed a weaker ability to assimilate xylose than was shown for *R. minuta*, with particularly poor growth when xylose was used as the only carbon source. Enhanced growth on arabinose compared to xylose was also observed by Sitepu *et al.*²⁵² They did however show better growth on D-arabinose than on L-arabinose. The growth productivity was similar for *R. minuta* when grown with cellobiose, fructose and lactose, but an increased assimilation of sucrose was observed for *R. glutinis*.

5.3.3 Effect of inhibitory compounds on the growth of *Rhodotorula sp.*

During lignocellulose hydrolysis, especially when acid catalysed, the sugars within the biomass will also degrade into numerous inhibitory products. Xylose degrades into furfural, whereas glucose degrades into 5-HMF. Both compounds are well understood as major fermentation inhibitors for ethanol production,²⁵⁰ being known to inhibit alcohol dehydrogenase, pyruvate dehydrogenase and aldehyde dehydrogenase.²⁵⁷ Acetic acid, levulinic acid and formic acid are the major organic acids formed during lignocellulose hydrolysis. Acetic acid is formed by hydrolysis of the acetyl group of hemicellulose linked to the lignin or cellulose matrix. Levulinic acid is formed following oxidation of mannose and glucose; formic acid can also be produced as a byproduct of this pathway. Formic acid is also the terminal oxidation product of xylose oxidation.^{250,258} These inhibitors are well known for their toxicity to microorganisms such as *S. cerevisiae*, generally in the order: acetic acid < levulinic acid < formic acid,²⁵⁹ but they tend to be less inhibitory to oleaginous yeasts.²⁶⁰ Cell survival and metabolism within a biomass hydrolysate environment is the first priority during the fermentation, as no product can be generated without sufficient growth. To assess the effect of these inhibitors on the growth of *R. glutinis* and *R. minuta*, the yeasts were cultured on a medium with 30 g/L glucose and either a low, medium or high level of the various inhibitors (Table 5.3). Multiple inhibitor concentrations were selected to correspond to the concentrations commonly present in various lignocellulose hydrolysates.²⁶¹⁻²⁶² The results were normalised to the control (30 g/L glucose with no additional inhibitors).

Table 5.3: Concentration of inhibitors used in the culturing of *R. minuta* and *R. glutinis* on glucose (30 g/L) in 96 well plates.

Inhibitor	Low value (mM)	Medium value (mM)	High value (mM)
Furfural	1	10	60
5-HMF	1	10	60
Acetic acid	10	60	200
Formic acid	10	60	200
Levulinic acid	10	60	200

R. glutinis still showed reasonable growth in the presence of furfural and 5-HMF, yielding around 65% of the growth of the control at the medium (10 mM) concentration. *R. glutinis* could also tolerate low (10 mM) loadings of organic acids, displaying the same pattern of toxicity as described above for *S. cerevisiae* (acetic- < levulinic- < formic acid).²⁵⁹ The tolerance to levulinic acid at the medium concentration (60 mM) was greater than both acetic and formic acid. This effect was also observed by Chen *et al.* in which the yeast strains that they tested (including *R. glutinis*) could tolerate double the concentration of levulinic acid compared to acetic- and formic acid.²⁵⁰ The growth of *T. cutaneum* was enhanced by the presence of levulinic acid.²⁵⁰ At high concentrations of organic acids, the growth of both *R. glutinis* and *R. minuta* used in this study was reduced substantially (Figure 5.5, a).

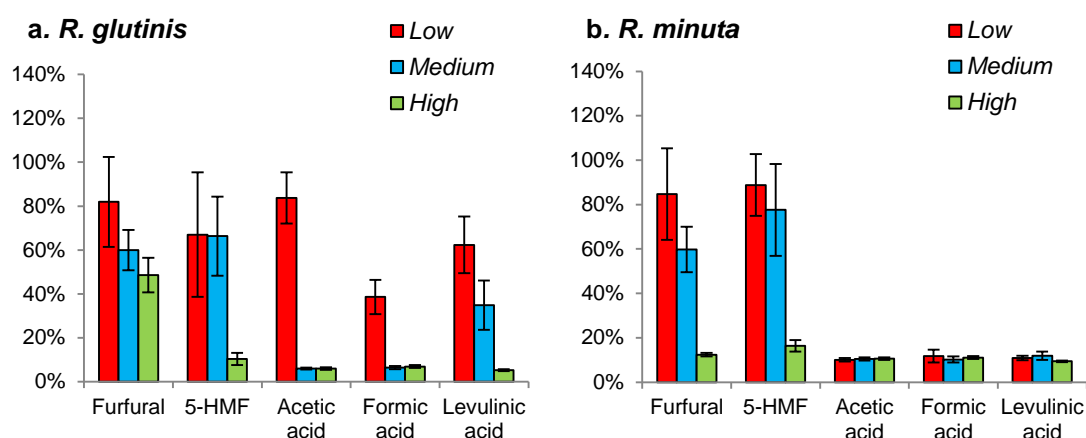


Figure 5.5: Biomass productivity from *Rhodotorula* sp. in the presence of common inhibitors present in depolymerised lignocellulose. The optical density measurement at 600 nm (OD_{600}) has been standardised to a percentage of the growth on glucose. The yeasts were cultured at 28 °C in 96 well plates over 120 h. The error bars represent the standard deviation from the mean where $n=6$. Low (red), medium (blue) and high (green) values represent 1, 10 and 60 mM for 5-HMF and furfural, and 10, 60 and 100 mM for the acids, respectively.

In comparison to *R. glutinis*, *R. minuta* demonstrates a similar tolerance to furfural and 5-HMF, yet the acidic inhibitors; acetic acid, formic acid and levulinic acid affected the growth substantially (Figure 5.5, b). This suggested that *R. minuta* may only be a suitable organism in scenarios where a gentle depolymerisation techniques have been used (e.g. enzyme hydrolysis), where there would be a low concentration of organic acids. Previous work demonstrated that *R. glutinis* can grow effectively on media containing 0.3 ml/L furfural and 0.26 g acetic acid/gram of sugar (equivalent to 4 mM furfural and 129 mM acetic acid) if xylose is used as the sugar substrate.⁵⁷ In contrast, when glucose was used as the substrate, Zhang *et al.* reported the specific growth rate to decrease from 0.31 h⁻¹ when growing *R. glutinis* on pure glucose to 0.13 h⁻¹ when the inhibitors were also present.⁵⁷ According to the results of this study, this effect is more likely due to the presence of the acetic acid rather than furfural. Similarly, a 35% reduction in growth rate was observed for *R. grammis* at a furfural concentration of 0.75 g/L, and was inhibited completely at 2.6 g/L.⁵⁶

Furfural is known to deactivate the mechanisms of cell replication, and cause damage to the vacuole and mitochondrial membrane.²⁵⁸ Recent work by Sitepu *et al.* suggested that *R. minuta* and *R. glutinis* have no tolerance to furfural at 0.5 g/L (5.2 mM) when glucose is used as the carbon source,²⁵² in contrast to the work presented here. Sitepu *et al.* also showed varying growth with differing concentrations of 5-HMF, demonstrating that *R. glutinis* could tolerate 5-HMF concentration up to 0.5 g/L (4 mM), whereas *R. minuta* exhibited delayed growth in concentrations up to 1 g/L (8 mM). Neither species could grow when 5-HMF was present at 2 g/L (16 mM). Sitepu *et al.* also tested acetic acid at 2.5 g/L (42 mM), in which they demonstrated no growth for either *R. glutinis* or *R. minuta*. While *R. glutinis* can tolerate low concentrations of acetic acid (10 mM), *R. minuta* growth was reduced substantially. These are however model studies, in which the five inhibitory compound were tested separately. In biomass hydrolysates, a mixture of sugars and inhibitory compounds are present and therefore there is the potential for a negative synergistic effect on the yeast growth.²⁵⁰ As such, the latter half of

this chapter focuses on the production of SCO from depolymerised biomass hydrolysates.

5.3.4 Hydrolysis of *Miscanthus giganteus*

Enzymatic hydrolysis of lignocellulosic feedstocks has been considered as the most promising approach for the saccharification of biomass streams as the mild pretreatment conditions reduce the amount of inhibitory compounds formed. However, using solely enzymes as the depolymerisation mechanism can result in low sugar yields as many plants have developed natural resistance to microbial breakdown that prevent the release of vital sugar sources.²³⁵ These can include natural inhibitors present in the biomass as well as microbial inhibitors released through biomass degradation as mentioned previously. In order to enable access to the hemicellulose and cellulose fibrils, pretreatment with acid and/or ammonia is used prior to saccharification.

Miscanthus giganteus is a herbaceous perennial crop that has attracted considerable interest in recent years as an energy crop due to an abundant source of lignocellulosic biomass.²⁶³ *Miscanthus* is one of four crops in the EU that has been identified as the most promising in terms of being a feedstock for biofuel production.⁸⁵ Compared to annual crops such as wheat or barley, *Miscanthus* offers enhanced environmental characteristics including lower resource input (water, fertiliser, pesticides), it does not require re-seeding and it can be grown on marginal land unsuitable for food crops. Furthermore, the deep-rooted structure helps to prevent soil erosion and increase soil fertility.²⁶⁴ In England, yields of up to 18.7 t/ha (dry matter) can be produced, with a natural cellulose content higher than other energy crops, thereby increasing the amount of glucose in the hydrolysate.²⁶⁵

The *Miscanthus* hydrolysates were kindly provided by Hussein *et al.* in the Dept. of Biology & Biochemistry, University of Bath using a combination of ammonia pretreatment followed by acid and enzymatic (cellulase) hydrolysis (Table 5.4). Due to the limited volume of these hydrolysates, only the growth and lipid productivity of *R. minuta* was assessed, as the growth of this yeast on depolymerised lignocellulosic feedstocks is less understood than that of *R. glutinis*.

Table 5.4: Hydrolysis methods for *Miscanthus giganteus* provided by Hussein *et al.*

	Hydrolysis method
1	Untreated <i>Miscanthus</i> hydrolysed with 1% H ₂ SO ₄ . Insoluble biomass hydrolysed with cellulases.
2	Untreated <i>Miscanthus</i> hydrolysed with 1% H ₂ SO ₄ . Soluble fraction hydrolysed with cellulases.
3	Pretreated (ammonia soaked) <i>Miscanthus</i> hydrolysed with 1% H ₂ SO ₄ . Insoluble biomass hydrolysed with cellulases.
4	Pretreated (ammonia soaked) <i>Miscanthus</i> hydrolysed with 1% H ₂ SO ₄ . Soluble fraction hydrolysed with cellulases.
5	Pretreated (ammonia soaked) <i>Miscanthus</i> hydrolysed with only cellulases
Control	RSM medium with 30 g/L glucose and 0.5 g/L (NH ₄) ₂ SO ₄

Sugars in the five *Miscanthus* hydrolysates were analysed by HPLC following hydrolysis with the CTEC-2 cellulases (Figure 5.6), and the total sugar concentration was quantified by the sum of the individuals sugars present in the hydrolysate (glucose, xylose, arabinose and galactose). Following acid hydrolysis on the untreated *Miscanthus* biomass, ~5 g/L of sugar was obtained in the soluble fraction (hydrolysate #2). Very little glucose was obtained by this method, suggesting that only the sugars within the hemicellulose were hydrolysed. Cellulase hydrolysis of the insoluble biomass following acid pretreatment yielded very small amounts of sugar suggesting that this was not a suitable method (hydrolysate #1). To achieve the highest sugar yields, pretreatment by ammonia soaking was important. While ammonia pretreatment did not increase the sugars obtained from the hemicellulose, nor the quantity of sugars in the soluble fraction (hydrolysate #4), it greatly improved the glucose yield from the insoluble biomass following ammonia soaking and acid pretreatment (hydrolysate #3). Ammonia pretreatment is known to cause expansion of the cellulose fibrils, enabling access for the cellulase enzymes to break down the glucose polysaccharides.²³⁶ However ammonium soaking alone did not yield as much sugar as when both ammonia and acid pretreatment were both used prior to enzyme hydrolysis (hydrolysates #5 and #3, respectively).

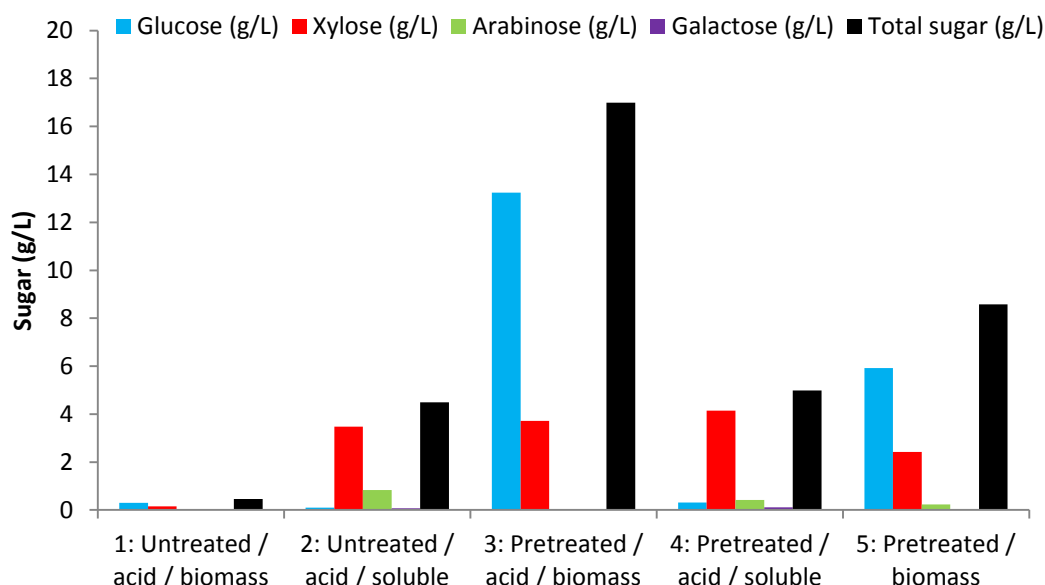


Figure 5.6: Quantification of the sugars resulting from five depolymerisation methods for *Miscanthus giganteus* hydrolysis. The biomass was used untreated or pretreated with ammonia soaking. Acid pretreatment with 1% (v/v) H_2SO_4 was also used for hydrolysates 1-4. Following pretreatment, the insoluble biomass or soluble fraction was hydrolysed with CTEC-2 cellulase mix in phosphate buffered saline. Sugar was quantified using HPLC and compared to known standards. The data represents a one-off scoping study.

5.3.4.1 *R. minuta* growth on *Miscanthus* hydrolysates

The time course experiments for *R. minuta* cultured on the five different *Miscanthus* hydrolysates are shown in Figure 5.7. In the cultures that produced biomass (hydrolysates 1, 2 & 4), no obvious lag time was observed, suggesting that the concentration of inhibitors within these hydrolysates was low. Aside from the control, all cultures reached stationary phase around 72 h, most likely due to the cultures assimilating all of the sugar present in the hydrolysates. Most notably, no biomass was produced from hydrolysates 3 and 5, which had the highest total sugar concentration of 17 g/L and 8.6 g/L, respectively. As there was growth on the other hydrolysates, this indicated that there was likely to be a high concentration of inhibitors resulting from the depolymerisation method. This illustrated the fine balance between sugar extraction and inhibitor formation. While Chen *et al.* reported the quantification of inhibitory compounds via HPLC,²⁵⁰ attempts to replicate this were unfruitful due to the inability to produce a suitable standard curve.

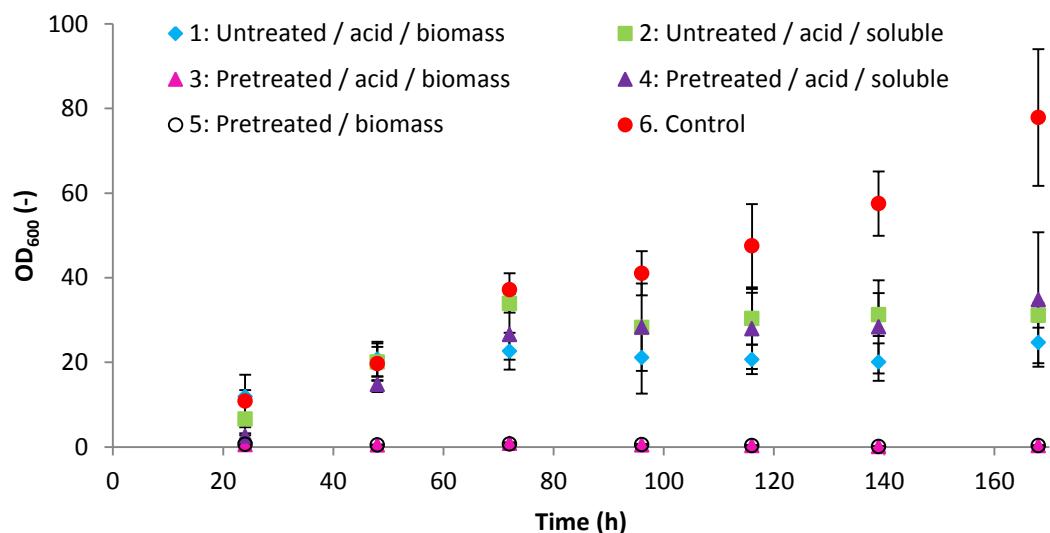


Figure 5.7: Optical density measurements at 600 nm (OD_{600}) for the growth of *Rhodotorula minuta* on depolymerised *Miscanthus giganteus* hydrolysate. The biomass was used untreated or pretreated with ammonia soaking. Acid pretreatment with 1% (v/v) H_2SO_4 was also used for hydrolysates 1-4. Following pretreatment, the insoluble biomass or soluble fraction was hydrolysed with CTEC-2 cellulase mix in phosphate buffered saline. All samples (20 ml per hydrolysate) were filtered and sterilised before culturing *R. minuta* at 28°C, 180 rpm for 168 h in 100 ml conical flasks. The error bars represent the standard deviation from the mean where $n=3$.

The greatest biomass yield (5.9 ± 0.58 g/L) was obtained from hydrolysate #4, in which ammonia-soaked *Miscanthus* was pretreated with acid and the soluble fraction hydrolysed with the cellulase enzymes (Figure 5.8). Biomass resulting from the untreated *Miscanthus* yielded 2.53 ± 2.19 g/L and 4.67 ± 0.40 g/L for the insoluble (hydrolysate #1) and soluble (hydrolysate #2) fraction, respectively. These were however considerably lower than the control culture in which 13.4 ± 0.67 g/L of biomass was produced. This is however most likely to be due to the *R. minuta* culture assimilating all of the available sugar in the culture medium. Indeed, for hydrolysates 1, 2 and 4, there was no sugar present in the supernatant following yeast growth. Lipid yields were also low for all of the cultures suggesting that the C/N ratio was not suitable for lipid accumulation, or more likely, that degradation of the lipid reserve occurred after exhaustion of the carbon source in the culture medium.¹⁷ While there are no published studies surrounding the growth of *R. minuta* on similar hydrolysates, *R. glutinis* grown on depolymerised rice straw hydrolysates yielded poor growth (3.58 g/L biomass, 5.74% (w/w) lipid)²⁵³ which are similar to the results presented here. However, in the same study biomass yields of

16.56 g/L with a lipid content of 28.59% (w/w) was observed for *R. glutinis* grown on hydrolysed *Populus euramericana* leaves. Hot sulphuric acid hydrolysis was used in both scenarios.²⁵³

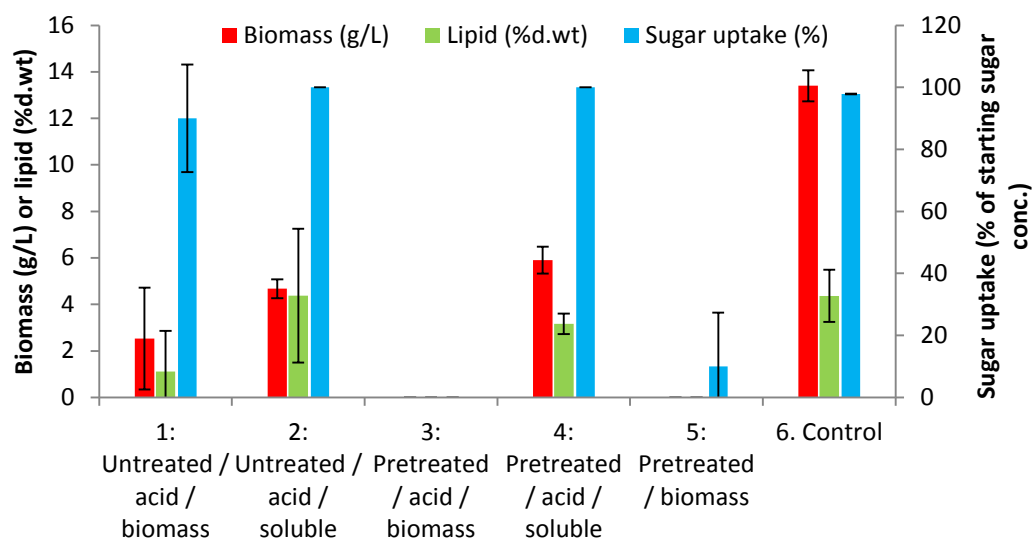


Figure 5.8: Biomass, lipid productivity and sugar uptake of *Rhodotorula minuta* grown on depolymerised *Miscanthus giganteus* hydrolysate. The biomass was used untreated or pretreated with ammonia soaking. Acid pretreatment with 1% (v/v) H_2SO_4 was also used for hydrolysates 1-4. Following pretreatment, the insoluble biomass or soluble fraction was hydrolysed with CTEC-2 cellulase mix in phosphate buffered saline. All samples were filtered and sterilised before culturing *R. minuta* at 28 °C, 180 rpm for 168 h. The error bars represent the standard deviation from the mean where n=3.

5.3.5 Food waste hydrolysis (enzymatic)

In 2012, 7 million tonnes of food and drink was wasted in UK households, equating to 260 kg per household per year.¹⁵⁸ By weight, it represents 19% of the food initially taken in to the home, and it is thought to equate to £12.5 billion across the UK.¹⁵⁸ Food waste is however a heterogenous mix, varying with location and season.²²⁸ As such, it is difficult to model for laboratory work. Using data available in: *Household Food and Drink Waste in the United Kingdom 2012*,¹⁵⁸ a representative food waste was developed based on the proportion of the weight of food waste in the UK (Figure 5.9). Drink waste was excluded from the representative sample as it usually exits the household via the sewerage system, rather than through the kerbside collections.¹⁵⁸

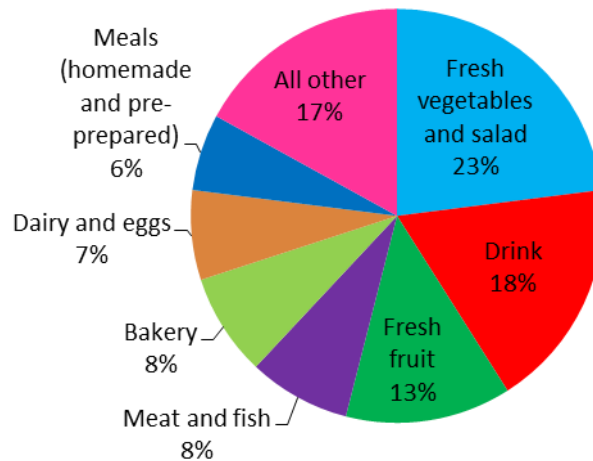


Figure 5.9 The proportion of the average weight of waste food and drink in the UK (data from Wrap, UK).¹⁵⁸

Yan *et al.* demonstrated that enzymatic hydrolysis of food waste is highly variable and as such hydrolysis optimisation is important to enhance productivity.²³² Out of eight variables tested, they identified enzyme loading (glucoamylase), incubation time, temperature and pH as the four most significant factors to the reducing sugar concentration obtained.²³² In this study, a central composite design was used to test the effects of these four variables on the cellulase (Celluclast®) degradation of food waste. The upper and lower variables of each level were selected based on the optimum conditions for enzyme activity according to data from the manufacturer, and previously published data (Table 5.5).^{232,238}

The total sugar concentration was the sum of the four most prevalent sugars as measured by HPLC (maltotriose, maltose, glucose and xylose). Table 5.5 shows the effect of these variables on total sugar concentration attained. The maximum sugar concentration ranged from 22.13 g/L – 35.08 g/L over the 30 experiments tested.

Table 5.5: Central composite design for the enzymatic hydrolysis of food waste relative to the total soluble sugar obtained and the biomass produced from *Rhodotorula sp.*

Run No.	Temp. (°C)	Time (h)	pH (-)	Cellulase load (%)	Total sugar (g/L)	<i>R. glutinis</i> biomass (g/L)	<i>R. minuta</i> biomass (g/L)
1	50	1	4.5	1	22.46	9.88	11.9
2	60	1	4.5	1	22.88	7.73	9.04
3	50	5	4.5	1	26.28	7.54	0
4	60	5	4.5	1	27.18	12.06	11.7
5	50	1	5.5	1	22.13	12.7	12.5
6	60	1	5.5	1	23.65	5.93	6.5
7	50	5	5.5	1	28.40	4.38	0
8	60	5	5.5	1	29.84	4.13	5.67
9	50	1	4.5	5	23.24	0	0
10	60	1	4.5	5	24.53	0	0
11	50	5	4.5	5	30.87	0	0
12	60	5	4.5	5	35.08	0	0
13	50	1	5.5	5	23.78	0	6.15
14	60	1	5.5	5	25.64	0	0
15	50	5	5.5	5	31.74	0	0
16	60	5	5.5	5	35.02	0	0
17	55	3	5	3	27.31	0	0
18	55	3	5	3	26.46	0	0
19	55	3	5	3	27.17	0	0
20	55	3	5	3	27.55	0	0
21	50	3	5	3	25.12	5.67	0
22	60	3	5	3	29.84	0	0
23	55	1	5	3	23.27	0	0
24	55	5	5	3	31.20	0	0
25	55	3	4.5	3	27.61	0	0
26	55	3	5.5	3	27.92	0	1.5
27	55	3	5	1	23.88	4.84	6.58
28	55	3	5	5	28.02	0	0
29	55	3	5	3	27.11	0	0
30	55	3	5	3	26.95	0	0
CONTROL					30.00	6.76	7.23

The correlation between temperature, time, pH and enzyme loading on the total sugar produced in the resulting hydrolysate was assessed. A regression analysis was performed to fit the response function and to predict the outcome using a radial basis function-multiquadratic (qRBF) response surface. The experimental responses, along with the predicted response obtained from the regression analysis are shown in Figure 5.10.

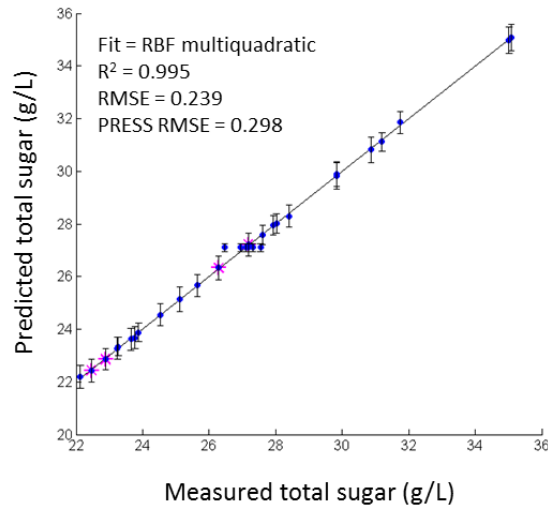


Figure 5.10: Response surface model fit for the total sugar released from the enzymatic hydrolysis of food waste. The error bars represent the spread of data observed for the repeated points. PRESS: Predicted sum square error; R^2 : Coefficient of determination; RBF: Radial basis function; RMSE: Root mean square error.

The displayed error bars indicated the close agreement between the predicted and measured response factors. The fit of the model was checked using the coefficient of determination, R^2 , which was calculated to be 0.995, indicating that 99.5% of the variability in the response could be explained by the model. The root mean squared error (RMSE) of the model was 0.239 g/L, with the predicted sum square error (PRESS) RMSE being 0.298 g/L. Furthermore, the analysis of variance (ANOVA) for the qRBF response surface are summarised in Table 5.6.

Table 5.6: Analysis of variance (ANOVA) for the response surface radial basis function (RBF)-multiquadratic.

Source	SS	Df	MS	RMSE	R^2
Regression	336.558	15.946	21.106	0.239	0.995
Error	0.745	13.054	0.057		
Total	338.36	29	0		

SS = sum of squares; DF = degrees of freedom; MS = mean square; RMSE = route mean square error.

5.3.5.1 Interaction between variables

The response surfaces based on the regression model showing the interaction of two variables are shown in Figure 5.11. A parallel surface of the plot with either of the axes indicates that no interaction exists between the two variables. This was

observed for pH, which had very little interaction with any of the other variables tested, and thus no influence on the total sugar obtained (Figure 5.11, c & d).

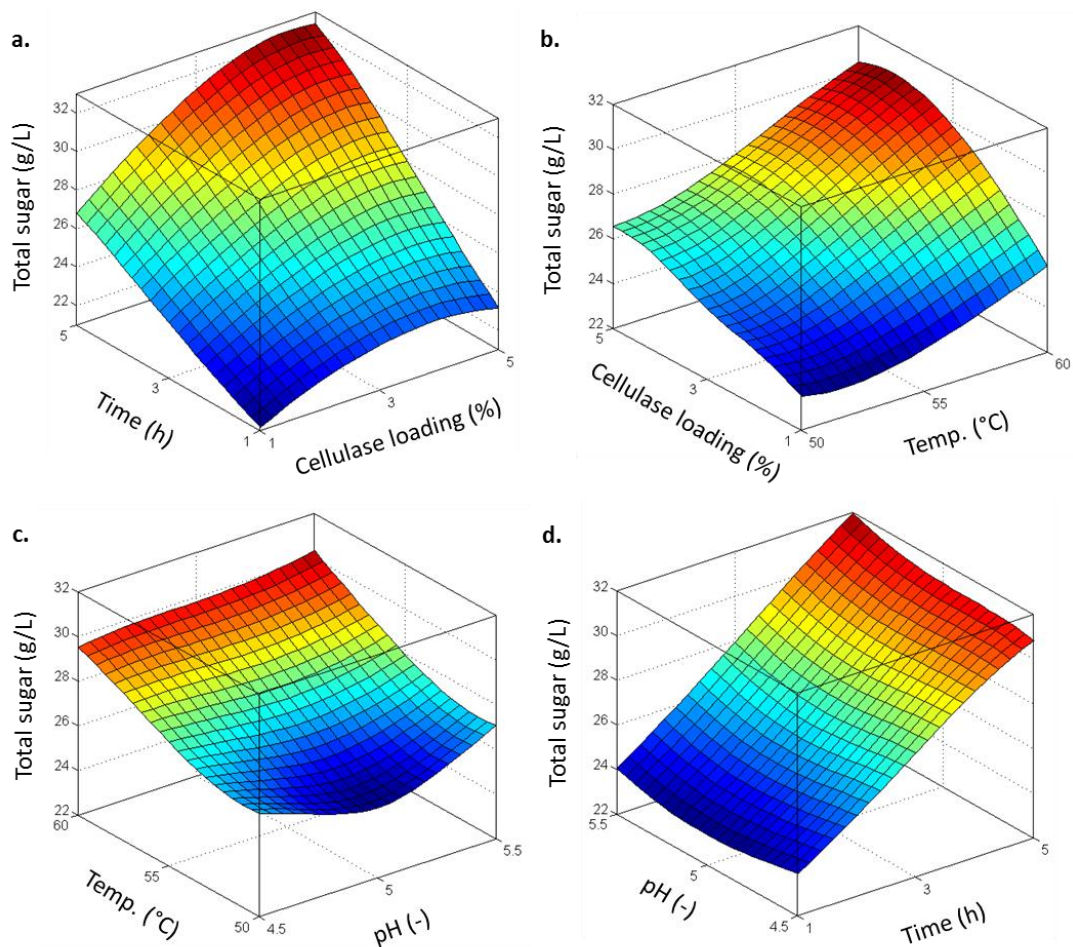


Figure 5.11: Response surface plots of the central composite design for the optimisation of enzymatic (cellulase) hydrolysis of food waste. Effect of: **a.** Time and cellulase loading; **b.** Cellulase loading and temperature; **c.** Temperature and pH; **d.** pH and time. Aside from the variables assessed, all other variables were constant at zero.

The greatest level of hydrolysed sugars was obtained at high levels of cellulase loading (3-5%), longer hydrolysis times (3-5 h) and high temperatures (60 °C). However, while it would be expected that higher sugar yields would promote yeast growth, no biomass was produced when *Rhodotorula sp.* was grown on the resulting high-sugar hydrolysates. Aside from two exceptions (run numbers 13 and 26), biomass was only produced when the enzyme loading was at 1%. In a similar situation to that observed for the *Miscanthus* hydrolysates, greater sugar yields resulting from increased enzyme loadings could cause increased concentrations of

inhibitors to be formed, especially following sterilisation by autoclaving. In a complex mixture such as food waste, chemical reactions like the Maillard reaction, in which the carbonyl group of the sugar reacts with the amino group of the amino acid to form a variety of complex molecules can occur.²⁶⁶ Regardless of this, the maximum sugar concentration obtained was only 35 g/L, which for a carbohydrate-rich feedstock such as food waste is not particularly high. While coupled enzymatic and thermochemical pretreatment can enhance the soluble sugar yield, Vavouraki *et al.* concluded that thermochemical pretreatment alone resulted in the highest sugar yields.²³⁷⁻²³⁸ Similarly, by undertaking a techno-economic analysis of the pretreatment methods for anaerobic digestion, Carballa *et al.* concluded that mechanical or thermochemical pretreatment was most suitable from an environmental perspective.²⁶⁷ Furthermore, it has been estimated that due to the excessive cost of enzyme use, enzymatic saccharification may be economically unsuitable.²³⁸ This is especially true for fungal cellulases, such as the one used in this study, which have a comparatively low activity. Research is therefore being directed towards thermophilic microbes which have an enhanced cellulase activity or enzyme cocktails which may result in higher yields of soluble sugar from the biomass feedstock.²¹

5.3.6 Food waste hydrolysis (thermochemical)

Acid-catalysed thermochemical pretreatment of biomass feedstocks has been explored as a simple and inexpensive method for the efficient saccharification of lignocellulosic feedstocks.²⁶⁸ The food waste in this study was depolymerised with 3% (v/w) H₂SO₄ and heated at 121 °C for 15 min using a modified method of Chi *et al.*⁸⁴ This method yielded a total soluble sugar concentration of 66 g/L, of which it was (w/w): 63% glucose, 25% xylose, 4.6% maltose and 7.4% maltotriose. The food waste hydrolysate (FWH) obtained by Chi *et al.* comprised of 51.2 g/L total sugar of which 92% was glucose.⁸⁴ Using this FWH, both *R. glutinis* and *R. minuta* were cultivated at 28 °C for 120 h (Figure 5.12 and Figure 5.13, respectively). Growth medium containing only glucose were used as the control cultures (Figure 5.14).

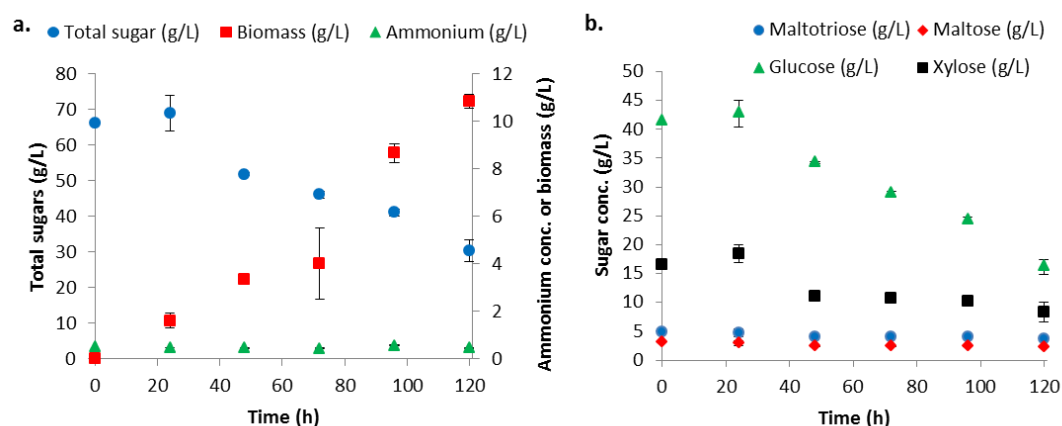


Figure 5.12: *Rhodotorula glutinis* growth on acid-hydrolysed food waste (FWH). **a.** Biomass production with ammonium and total sugar uptake; **b.** Uptake of the individual sugars present in food waste as analysed by HPLC. The food waste was hydrolysed with 3% (v/w) H_2SO_4 at 121 °C for 15 min. The biomass was filtered from the hydrolysate and neutralised to pH 6.5 with NaOH. *R. glutinis* was cultured on 100 ml of FWH in 250 ml Erlenmeyer flasks at 28 °C for 120 h, 180 rpm with 10 ml samples removed every 24 h. The error bars indicate the standard deviation from the mean where $n=3$.

A biomass yield of 8.06 g/L was observed with *R. glutinis* cultivated on the FWH. This is slightly lower than Chi *et al.* who yielded 10 g/L from *R. glutinis* cultivated on food waste. The proportion of glucose within the FWH used in this study was however lower than that of Chi *et al.*, being only 63% glucose and 25% xylose. Very little sugar was taken up during the first 24 h of *R. glutinis* growth suggesting that a lag phase was present, although this was not apparent in the biomass produced at that time (Figure 5.12, a). After this point, the concentration of glucose and xylose decreased simultaneously, supporting the data presented previously that this strain of *R. glutinis* does not exhibit a diauxic growth pattern. However, after 42 h the xylose concentration remained stable around 10 g/L and only glucose was assimilated. Maltose and maltotriose were not utilised at all throughout the growth period.

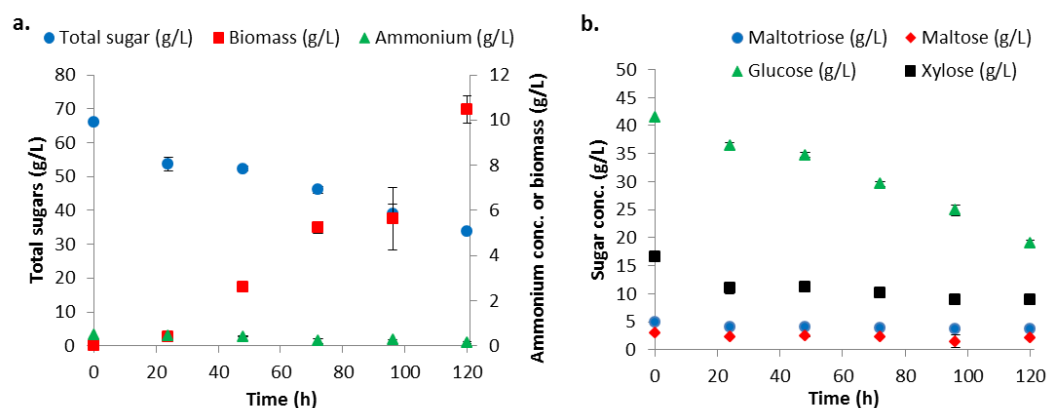


Figure 5.13: *Rhodotorula minuta* growth on acid-hydrolysed food waste (FWH). a. Biomass production with ammonium and total sugar uptake; b. Uptake of the individual sugars present in food waste as analysed by HPLC. The food waste was hydrolysed with 3% (v/w) H_2SO_4 at 121 °C for 15 min. The biomass was filtered from the hydrolysate and neutralised to pH 6.5 with NaOH. *R. minuta* was cultured on 100 ml of FWH in 250 ml Erlenmeyer flasks at 28 °C for 120 h, 180 rpm with 10 ml samples removed every 24 h. The error bars indicate the standard deviation from the mean where $n=3$.

When *R. minuta* was cultivated on the FWH, a clear lag phase was observed in the first 24 h of growth (Figure 5.13, a), which was not observed in the control culture (Figure 5.14, b). Conversely to *R. glutinis*, both glucose and xylose were assimilated from the start of the fermentation period, although the uptake of xylose ceased after the first 24 h, after which only glucose was assimilated (Figure 5.13, b). Maltose and maltotriose were not utilised at all during the culture with *R. minuta*. For the enzymatic hydrolysis of food waste, higher sugar yields prevented yeast growth, most likely due to the formation of inhibitory compounds. Even though the sugar yields resulting from the acid hydrolysis of food waste (FWH) were considerably higher at 65 g/L, more biomass was produced by the yeast growing on the FWH compared to the control (Figure 5.15).

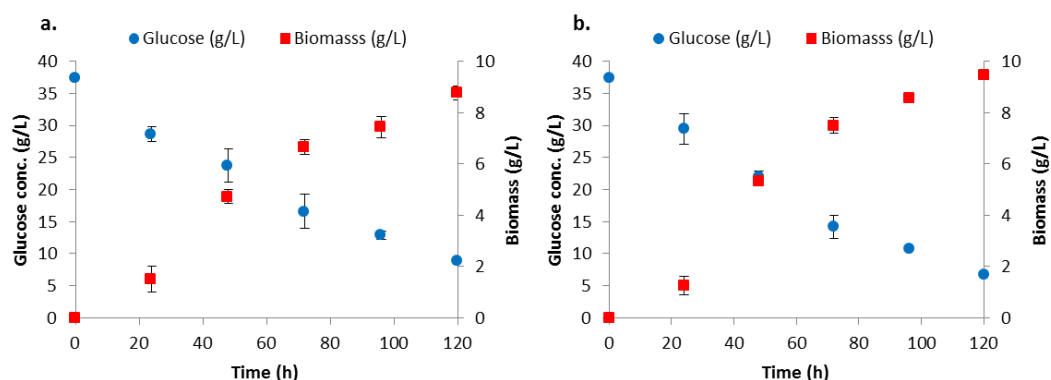


Figure 5.14: Biomass and glucose uptake for the control cultures of: a. *Rhodotorula glutinis*; and b. *Rhodotorula minuta*. The RSM medium was supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$. The cultures were grown for 120 h at 28 °C, 180 rpm in 250 ml Erlenmeyer flasks. The biomass yield was quantified from the freeze-dried biomass resulting from 10 ml of culture medium. High performance liquid chromatography was used to quantify the glucose uptake. The error bars indicate the standard deviation from the mean where n=3.

Alkaline detoxification (overliming) of pretreated lignocellulose has been used previously as an effective method of removing inhibitors from the hydrolysate.²⁶⁸ This process commonly utilises calcium hydroxide ($\text{Ca}(\text{OH})_2$), but other types of alkali can yield hydrolysates of similar fermentability.²⁶⁹ While the mechanism of this detoxification is still unclear, it is believed to be due to the precipitation of toxic substances.²⁷⁰ Indeed, a black precipitate was observed on the addition of concentrated sodium hydroxide (NaOH) to the acid-hydrolysed food waste hydrolysate in this study. However, Van Zyl *et al.* observed a complete lack of fermentation with *Pichia stipitis* when acid-hydrolysed bagasse was neutralised with NaOH instead of $\text{Ca}(\text{OH})_2$, suggesting that the sodium cations failed to remove the inhibitors in that case.²⁷⁰ In contrast, Alriksson *et al.* produced a hydrolysate with similar fermentability with *S. cerevisiae* when Norway spruce (*Picea abies*) was hydrolysed with dilute acid and detoxified with NaOH when compared to a synthetic sugar control.²⁶⁹ The process of overliming can however cause the soluble sugars in the hydrolysate to also precipitate and thus have a negative effect on the overall yield.²⁶⁸

5.3.6.1 Biomass and lipid yields for *Rhodotorula sp.*

Both *Rhodotorula sp.* yielded similar biomass yields of 10.84 ± 0.29 g/L for *R. glutinis* and 10.46 ± 0.60 g/L for *R. minuta* when cultivated on the food waste hydrolysate. This was slightly higher than ~ 9 g/L that was obtained for the respective control cultures (Figure 5.15).

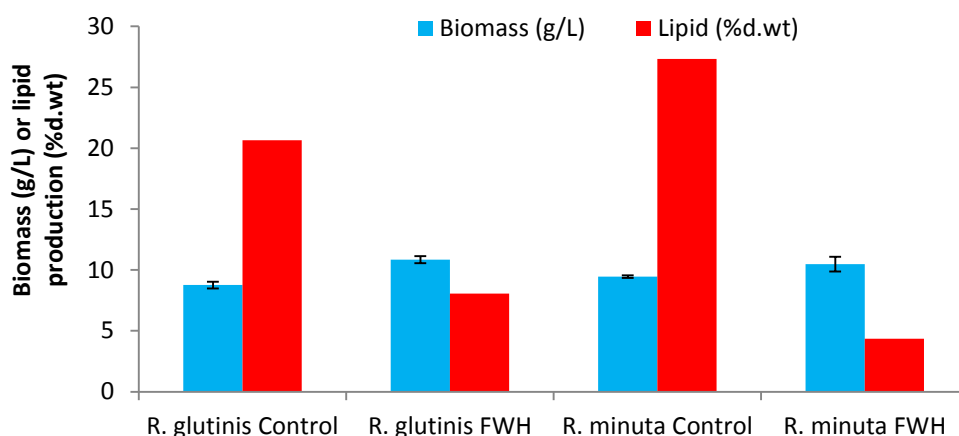


Figure 5.15: Biomass and lipid productivity of *Rhodotorula sp.* grown on food waste hydrolysate (FWH). The RSM medium supplemented with 30 g/L glucose and 0.5 g/L $(\text{NH}_4)_2\text{SO}_4$ was used as the control. The cultures were grown for 120 h at 28 °C, 180 rpm in 250 ml Erlenmeyer flasks. The resulting biomass was freeze dried before the lipid was extracted and transesterified from 0.1 g biomass with 2:1 CHCl_3 :MeOH and 0.1 g H_2SO_4 . The error bars represent the standard deviation from the mean where $n=3$.

The biomass coefficients (gram of biomass per gram of assimilated sugar) for *R. glutinis* and *R. minuta* were 0.30 g /g and 0.33 g/g, respectively. These were comparable to the control cultures which both yielded biomass coefficients of 0.31 g/g. The lipid yields however varied considerably. For the control culture of *R. glutinis*, the dry weight contained 20% lipid which decreased to 8% (d.wt) when the food waste hydrolysate was used as the control. This yielded lipid coefficients (gram of lipid per gram of sugar assimilated) of 0.064 g/g and 0.024 g/g for the control culture and FWH, respectively. Similarly, the control culture of *R. minuta* contained 27% (d.wt) lipid, whereas the culture grown on food waste hydrolysate only yielded 4% (d.wt) lipid. This resulted in lipid coefficients of 0.084 g/g and 0.014 g/g for the control and FWH, respectively. This is most likely due to a low C/N ratio present in the food waste, which may suggest that a second, lipid-producing step with only a carbon source may be required in the process to enhance lipid yields. The biomass

and lipid coefficients for the control cultures in this study were also comparable to the data presented in Chapter 3.

The lipid profiles obtained from all of the cultures were typical of what has been presented previously, consisting of palmitic- (16:0), steric- (18:0), oleic- (18:1) and linoleic acid (18:2). The relative proportions of these lipids did however vary between the yeast cultivated on food waste and solely on glucose (Table 5.7). The oil resulting from the food waste hydrolysate had a much lower proportion of saturated acids compared to the culture on glucose, being 24.7% saturated and 14.6% saturated for *R. glutinis* and *R. minuta*, respectively for the FWH. For the control cultures this increased to 27.7% and 27.6%, for *R. glutinis* and *R. minuta*, respectively. This suggested that a component present in the FWH was decreasing the activity of the desaturase enzymes present in the yeast cells.

Table 5.7: Percentage composition for the fatty acid profile for *Rhodotorula sp.* cultivated on food waste hydrolysate (FWH) or glucose as the sole carbon source.

Fatty acid	<i>R. glutinis</i> FWH	<i>R. glutinis</i> control	<i>R. minuta</i> FWH	<i>R. minuta</i> control
16:0	20.6	21.9	13.4	22.0
18:0	4.1	5.8	1.2	5.6
18:1	63.8	48.1	66.6	48.7
18:2	11.4	24.2	18.8	23.6

Most notably, the level of oleic acid (18:1) increased considerably when the yeast was grown on food waste hydrolysate. 63.8% and 66.6% (18:1) was produced from food waste compared to 48.1% and 48.7% (18:1) obtained when glucose was the sole carbon source for *R. glutinis* and *R. minuta*, respectively (Table 5.7). The oil produced from both *Rhodotorula sp.* therefore had a higher 18:1 content than that typically found in rapeseed oil (~60%),¹⁶ used for the production of biodiesel in the EU. As described in detail in Chapter 3, transesterification of the *Rhodotorula* oil would produce a fuel with enhanced physical properties, compatible with European (EN 14-214) and US (ASTM D6751-08) specifications for biodiesel.¹⁶

5.4 Conclusion

This chapter presented the cultivation of *Rhodotorula sp.* on a range of sugars and inhibitors present in biomass hydrolysates, as well as the growth on two biomass feedstocks; a fast-growing perennial grass, *Miscanthus* and household food waste. The *Miscanthus* hydrolysate was deemed to be an unsuitable resource for SCO production from *R. minuta*, due to the poor growth and lipid yield obtained. In contrast, thermochemically-hydrolysed food waste offers great potential for valorisation from *Rhodotorula sp.*, producing an oil that upon transesterification, would result in a biodiesel with enhanced physical properties, suitable as a drop-in fuel for the road-transport industry.

CHAPTER 6:

LIPID EXTRACTION & ENERGY USE ASSESSMENT

“Energy is liberated matter, matter is energy waiting to happen.”

Bill Bryson

6.1 Preamble

In this final chapter, the energy needed to produce yeast oils will be assessed, with a particular focus on the extraction and subsequent conversion into biodiesel. This work was published in the journal *Energy* (see below and Appendix C). The overall process of producing lipid from waste food will then be compared to producing methane from the same resource.

C. J. Chuck, D. Lou-Hing, R. Dean, L. A. Sargeant, R. J. Scott, R. W. Jenkins. **2014**. Simultaneous microwave extraction and synthesis of fatty acid methyl ester from the oleaginous yeast *Rhodotorula glutinis*. *Energy*. 69, 446-454.

6.2 Introduction

During the production of single cell oils, there are a variety of energy-intensive steps that can hinder the adoption for large scale production. These include the cultivation of the microorganisms, separation of the biomass from the culture medium, drying the harvested biomass, the extraction of the oil from the cell and the subsequent conversion to the final product.²⁷¹ In order for the process to be economically viable, it is imperative that lower-energy techniques are developed for each step of the process. Here, the extraction of the oil from the cell matrix was explored.

6.2.1 Extraction techniques

Lipid extraction techniques for yeast and microalgae are very similar and thus comparisons between the two can be made, though harsher techniques or conditions are generally required for microalgae due to the composition of the cell.²⁷¹ Microalgae have a much more substantial cell wall than yeast cells, making the latter easier to fragment to extract the intracellular lipid.

6.2.1.1 Solvent extraction

Organic solvents are commonly used to extract lipid from microbial biomass and can include hexane, methanol, ethanol, chloroform and diethyl ether.²⁷² The rate of extraction is influenced by the particle size, the type of solvent, temperature and agitation.¹⁰³ One of the most widely used methods was developed by Bligh and

Dyer, which uses chloroform and methanol in a 2:1 volumetric ratio with Soxhlet extraction glassware.¹⁵⁶ This process uses solvent evaporation, followed by subsequent condensation and percolation cycles to extract the lipid into the organic phase. When comparing five different organic solvent mixtures, Lee *et al.* concluded that extraction with chloroform/methanol extracted the highest oil yield from the microalga, *Botryococcus braunii*.²⁷³ Chloroform is however highly toxic, causing concerns surrounding its use. Solvent extraction using n-hexane has been shown to be more selective towards the extraction of neutral lipids compared to chloroform/methanol.¹⁰⁰ This, alongside its low cost and boiling point, makes it the most common solvent type used within industry, in spite of its hazardous nature.^{103,274} It also readily separates into two layers when water is added, thereby improving downstream separations. Achten suggested that solvent extraction for *Jatropha curcas* was only commercially viable at a large-scale production of more than 50 tonnes of biodiesel per day.^{103,275}

The efficiency of the extraction method can also be influenced by the polarity of the solvent used. Lewis *et al.* demonstrated that the sequence of solvent addition can influence the extraction yields.²⁷⁶ When extracting from freeze-dried biomass, adding the solvents in order of increasing polarity (chloroform, methanol and water) yielded the greatest results. They propose that this was due to the non-polar solvents weakening the interactions between the lipid and the cell wall to then enable the dissolution of the lipids into the organic layer.²⁷⁶ This is especially important when extracting oil from wet biomass as the water can form a shell around the cell, preventing the necessary contact between the chloroform and the lipid.

6.2.1.2 Alternative techniques

Cryogenic grinding, supercritical fluids, pressurised liquids, acid/base treatment, enzyme lysis or other mechanical disruption techniques have also been reported as methods to extract lipids from the cell.²⁷⁷ Sonication has also been widely used as a method for disrupting microbial cells which uses a cavitation effect to crack the cell wall/membrane. Bead beating has been used on a laboratory and industrial scale to cause direct damage to cells using fine beads and high-speed mechanical spinning.

More novel methods, such as the *milking* of microalgal cells have also been reported for the extraction of oil from live cells.²⁷⁸ However, Lee *et al.* found that microwave extraction was the most effective method for extraction when comparing autoclaving, bead-beating, microwave-irradiation and sonication from three species of microalgae (*Botryococcus sp.*, *C. vulgaris* and *Scenedesmus sp.*).²⁷⁷

6.2.1.3 Microwave extraction

In contrast to conventional heating methods, in which heat transfer is by convection and conduction, microwaves cause direct generation of heat within the material, which can enhance the rate of reaction. Reactions using microwave-assisted heating have been shown to proceed up to 24-times faster than the same method using conventional heating methods.²⁷⁹ First used for extraction in the 1980s, microwave-assisted extraction is a fast and efficient extraction method used for solid-liquid extraction.²⁸⁰

The microwave region of the electromagnetic spectrum encompasses frequencies between 0.3 to 300 GHz, but microwaves generally operate at 2.45 GHz to avoid interferences with radio communications.²⁸⁰ For this type of extraction, the solvent used must possess a dipole moment to be able to absorb the microwave irradiation. Hexane is a non-polar solvent (the dielectric constant, ϵ , is 1.88 (-)) and thus unsuitable for microwave extraction.²⁷¹ In contrast, the method of Bligh and Dyer,¹⁵⁶ using a mixture of chloroform and methanol (2:1, (v/v)) is much more polar with dielectric constants of 4.81 (-) and 32.70 (-), respectively making it a more suitable solvent system for use in the microwave extraction. When exposed to microwave irradiation, a molecule with a dipole moment will realign with the applied electric field, however this is not possible when the field is oscillating. At 2.45 GHz, the oscillations occurs at 4.9×10^9 times per second.²⁸⁰ This continued movement causes friction within the sample and thus generates a large amount of heat. Heat is also generated if charged species are present, as the molecules collide when moving back and forth towards the electric field. The combination of lipid and methanol within the sample means that both ionic and polar molecules are present and thus very efficient heating is obtained.^{27a} Balasubramanian *et al.* showed that for cellular extraction, the immediate temperature rise and pressure

increase within the matrix triggered by microwave heating causes an electroporation effect within the structure of the cell wall (Figure 6.1). As such, solutes within the biomass (e.g. lipids) are able to pass freely into the organic phase,²⁷⁴ enabling a high recovery of the target molecules.²⁸¹

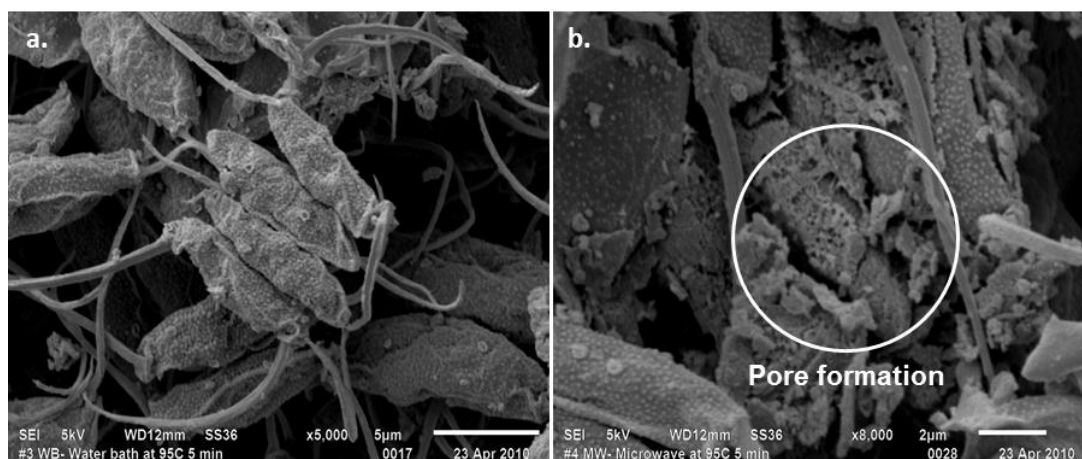


Figure 6.1: Scanning electron microscopy images illustrating the effect of conventional and microwave heat treatment on microalgal cells (*Scenedesmus obliquus*). a. Water bath, 95 °C, 5 min (5000x magnification); b. Microwave, 95 °C, 5 min (8000x magnification). The immediate increase in temperature and pressure causes an electroporation effect within the cell wall resulting in the formation of pores.²⁷⁴

Whilst this technique still relies on the use of solvents, the volume is often markedly less than traditional solvent extraction, and the pressure produced within the microwave chamber can be varied depending on the volume and the boiling point of the solvent.^{272,280}

6.2.1.4 *In-situ* extraction and transesterification

To convert the lipids into biodiesel, or to be able to analyse the lipid profile by gas chromatography, the lipids must be transesterified into their corresponding fatty acid methyl esters (FAME). This is essential for determining the quantity and physical properties of the lipid and/or biodiesel. Microbial oil transesterification can either take place directly, as the oil is being extracted from the cell, or indirectly, with the transesterification process occurring on purified microbial oil.²⁸² Direct transesterification has been used since the 1980s, when a similar method was used to prepare the FAME profile for yeast identification before ribosomal DNA analysis gained in popularity, but indirect transesterification is more common in industry.

The transesterification reaction can either be acid or base catalysed. Base-catalysed transesterifications are faster than the acid-catalysed equivalent, but the likelihood of soap formation is high, especially if there are free fatty acids (FFA) present in the extraction mixture. This is due to the saponification of the fatty acid in the presence of water.²⁸³ Base catalysis is therefore more suitable for indirect transesterifications when there is very little water present in the oil. Acid catalysis avoids soap formation, but generally longer reaction times and higher temperatures are required in order to reach the same FAME yields.^{279,284} For example, a Brønstead acid catalyst, such as H_2SO_4 is approximately 4000 times slower than sodium methoxide at 65 °C.²⁸⁵ For the direct transesterification of oil from microbial biomass, acid catalysis is more suitable due to the presence of water and FFA in the cell alongside the stored triacylglycerides. The FFA content within the cell can also increase post-fermentation when lipases present in the cell following fermentation can further hydrolyse the oil within the cell, increasing the FFA content.⁹⁰

6.2.2 Aims of the chapter

The aim of this chapter is to energetically assess the feasibility of SCO production. Firstly, this will involve experimentally assessing microwave extraction for the removal of oil from the yeast cells. This will be achieved using an energy return on investment methodology, comparing reaction time, temperature and catalyst concentration on the resulting energy in the oil obtained. The latter half of the chapter aims to produce a theoretical energy balance of two waste-to-energy routes, comparing SCO production from food waste to that of anaerobic digestion of food waste to produce methane.

6.3 Results and discussion

6.3.1 Soxhlet extraction

Soxhlet extraction has been widely used as a laboratory method for the extraction of lipid for over a century.²⁸⁶ However, this method is time consuming, requiring separate extraction and transesterification steps, and large quantities (~500 ml) of solvent per sample. In this study, the yeast lipid was extracted using Soxhlet glassware over a range of reaction times in order to achieve a theoretical maximum oil yield in the yeast biomass being studied (Figure 6.2). Once extracted, the resulting oil was transesterified at reflux in the presence of excess methanol and 10 wt.% H₂SO₄ as the catalyst. The resulting biodiesel was quantified by GC-MS. After 30 min extraction time, only 0.43% of the lipid was extracted, which increased to 22% after 1 h. To obtain complete lipid recovery (32%) 4 h of extraction was required. After this point, the lipid level as well as the lipid profile remained constant, which suggested that no degradation of the lipid was taking place over the longer reaction times. The lipid profile was consistent with that expected of *R. glutinis*, with mostly C₁₆ and C₁₈ fatty acids observed. Approximately 5% of the lipid was C₁₂ and C₁₄ lipids (Figure 6.2, b).

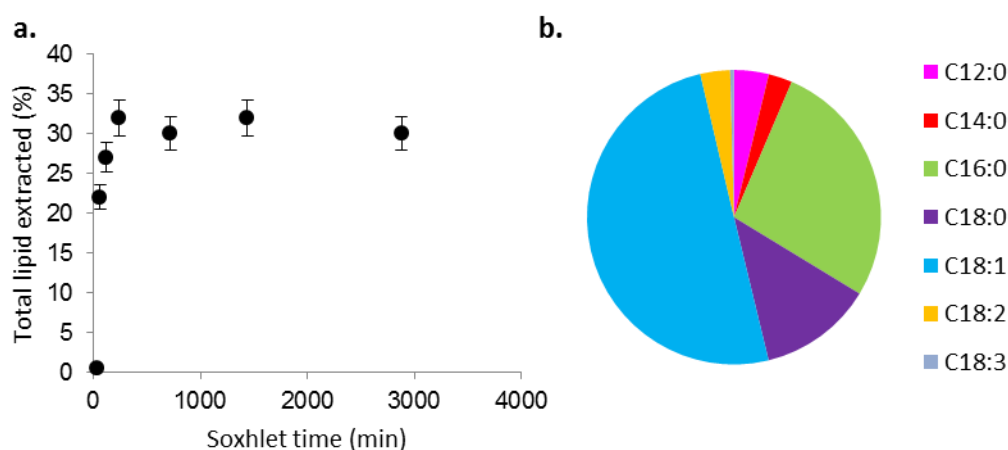


Figure 6.2: Extraction of lipids from freeze-dried *Rhodotorula glutinis* biomass using Soxhlet extraction. a. Total lipid extracted as a function of time; b. Average lipid profile for all of the Soxhlet extractions. Extractions took place with 50 ml of 2:1 CHCl₃:MeOH at reflux with 0.1 g of freeze-dried *R. glutinis* biomass in Soxhlet apparatus. The solvent was removed under vacuum and the lipid transesterified using 100 µl conc. H₂SO₄ in MeOH before being analysed by GC-MS and compared to known standards.

6.3.2 Microwave extraction

Microwave technology has been increasingly used as an effective method for cell disruption in microalgae,²⁸⁰ as it offers the potential for fast, high-throughput extraction. Small changes in the temperature and extraction time using microwave technology have been shown to significantly influence the quantity of oil extracted.²⁷⁴ In this study, an Anton Paar 300 monowave was used to extract the lipid from *R. glutinis* biomass. In order to explore the effects of reactions times, temperature and catalyst loading on the overall lipid yield, three reaction times: 0.5, 5, 10 and 20 min were selected at three reaction temperatures: 80, 100 and 120 °C, with four catalyst loadings: 1, 10, 25 and 100 wt.% in relation to the biomass. Temperature, pressure and wattage were recorded throughout the reaction, with the latter two variables measured, but not controlled (Figure 6.3).

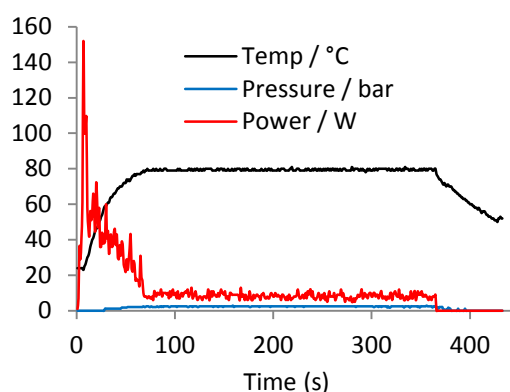


Figure 6.3: Reaction conditions for the automated Anton Paar monowave 300, showing the pressure, temperature and power of the microwave extraction of *Rhodotorula glutinis* oil with 100 wt.% H_2SO_4 at 80 °C over 5 min. The *in-situ* extraction and transesterification took place in a 10 ml microwave vials containing 0.1 g of the freeze-dried *R. glutinis* biomass and 6ml of 2:1 CHCl_3 :MeOH. The data was measured automatically every 0.5 s using the in-built monowave software.

The set temperature of the sample was reached within 80 s of the reaction commencing, during which time the largest amount of energy was required. The temperature was held for the length of the reaction, during which the pressure remained consistently around 2 bar. Once the reaction time had elapsed, the reaction vessel was cooled to 50°C with compressed nitrogen.

For all reactions, 6 ml of 2:1 CHCl_3 :MeOH was used with 0.1 g freeze-dried *R. glutinis* biomass. The effect of variation in the acid catalyst (H_2SO_4) loading on the

conversion of the yeast oil was established by ^1H NMR spectroscopy (Figure 6.4). For a triacylglyceride, the ^1H NMR spectrum shows the resonance corresponding to the multiplet of the five protons in the glyceride backbone between δ 4.0 – 4.2 ppm (Figure 6.4, a). Once fully transesterified, this multiplet disappears and the methoxy group of the fatty acid methyl ester (FAME) is observed at δ 3.6 ppm (Figure 6.4, b). By comparing the integral values of these two peaks, the conversion of lipid into biodiesel (FAME) can be calculated.¹⁶¹

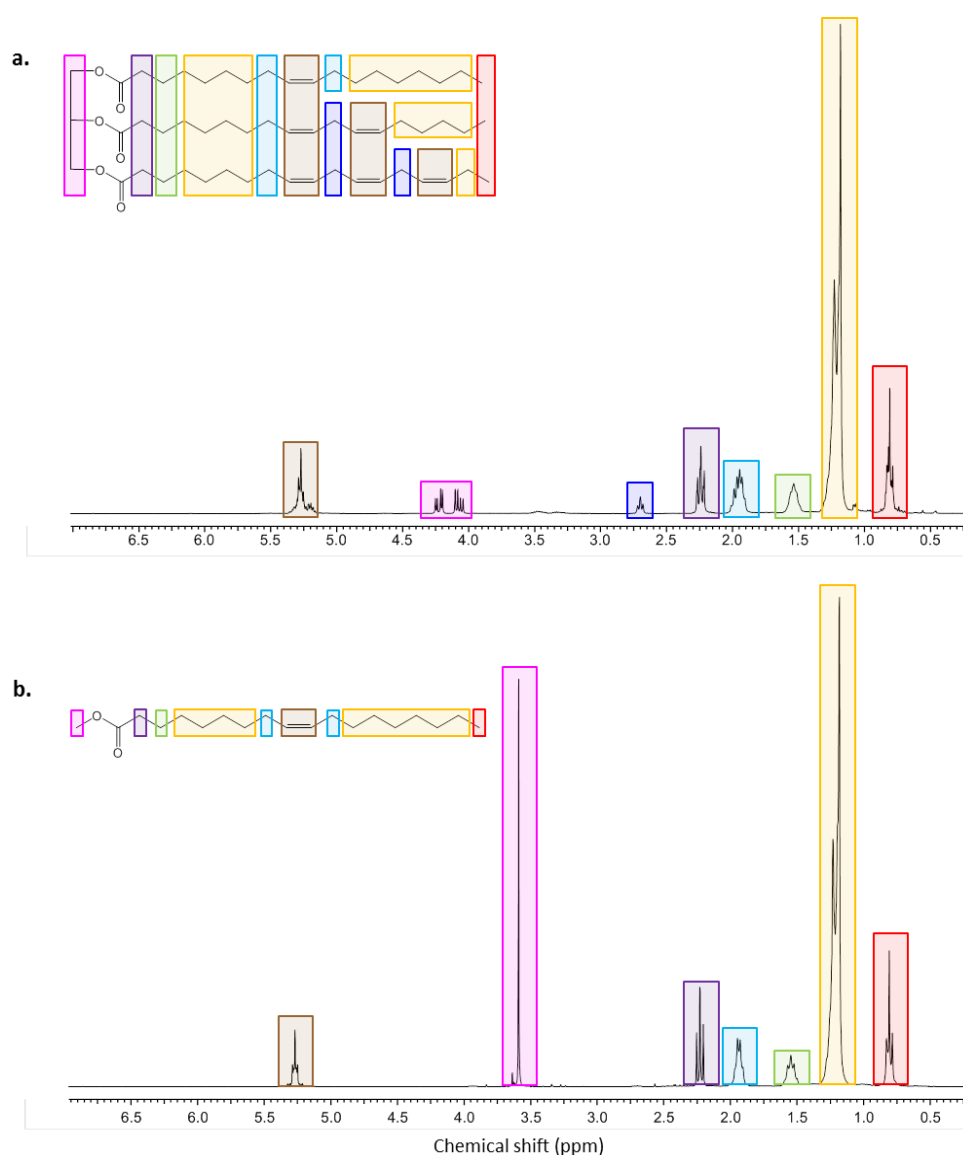


Figure 6.4: ^1H NMR spectrum (300 MHz) of: **a. *Rhodotorula glutinis* oil;** and **b. *Rhodotorula glutinis* fatty acid methyl ester.** The *R. glutinis* oil was transesterified with 100 wt.% H_2SO_4 catalyst, in 2:1 CHCl_3 :MeOH at 80 °C for 16 h. The methanol and acid catalyst were removed by washing with water, before the remaining chloroform was removed under vacuum and the samples dissolved in CDCl_3 for analysis. The resonance associated with the different proton environments have been colour coded.

6.3.2.1 Effect of catalyst loading on FAME yield

The catalyst loading of 1 wt.% was completely ineffective at converting any extracted lipids into FAME, with a maximum of 7% of glyceride being converted into biodiesel (Figure 6.5, a & b). By increasing the catalyst loading to 10 wt.%, a maximum conversion of 56% was observed at the longest reaction times and highest temperature (Figure 6.5, c).

These results were in accordance with Wahlen *et al.*, observed a maximum FAME yield of approximately 15% at 80 °C for 16 min with 5 wt.% H₂SO₄ using microwave irradiation for the transesterification of purified soybean oil.¹⁶² Once the level of catalyst loading was increased to 25 wt.%, 95% conversion was yielded at 120 °C over 5 min (Figure 6.5, d). At lower temperatures (80 and 100 °C), approximately 90% conversion was reached over the longest reaction times. Once the catalyst loading was increased to 100 wt.%, greater than 98% conversion was obtained at all temperatures where the reaction time was 5 minutes or longer (Figure 6.5, e). Therefore, in order to achieve sufficient conversion yields in a reaction time of 5 min or less, higher levels of catalyst loading must be used. This is in accordance with Perin *et al.* who reported that for complete esterification of castor oil using microwave irradiation, 10 wt.% catalyst and 30 min was required.²⁸⁷ These reactions are however substantially faster than the equivalent extractions using conventional heating methods. For the direct transesterification of microbial oil from *R. toruloides*, *L. starkeyi* and *M. isabellina*, Liu and Zhao achieved yield of up to 98% at 70 °C with an acid catalyst, when the reaction time was 20 h.²⁸⁸

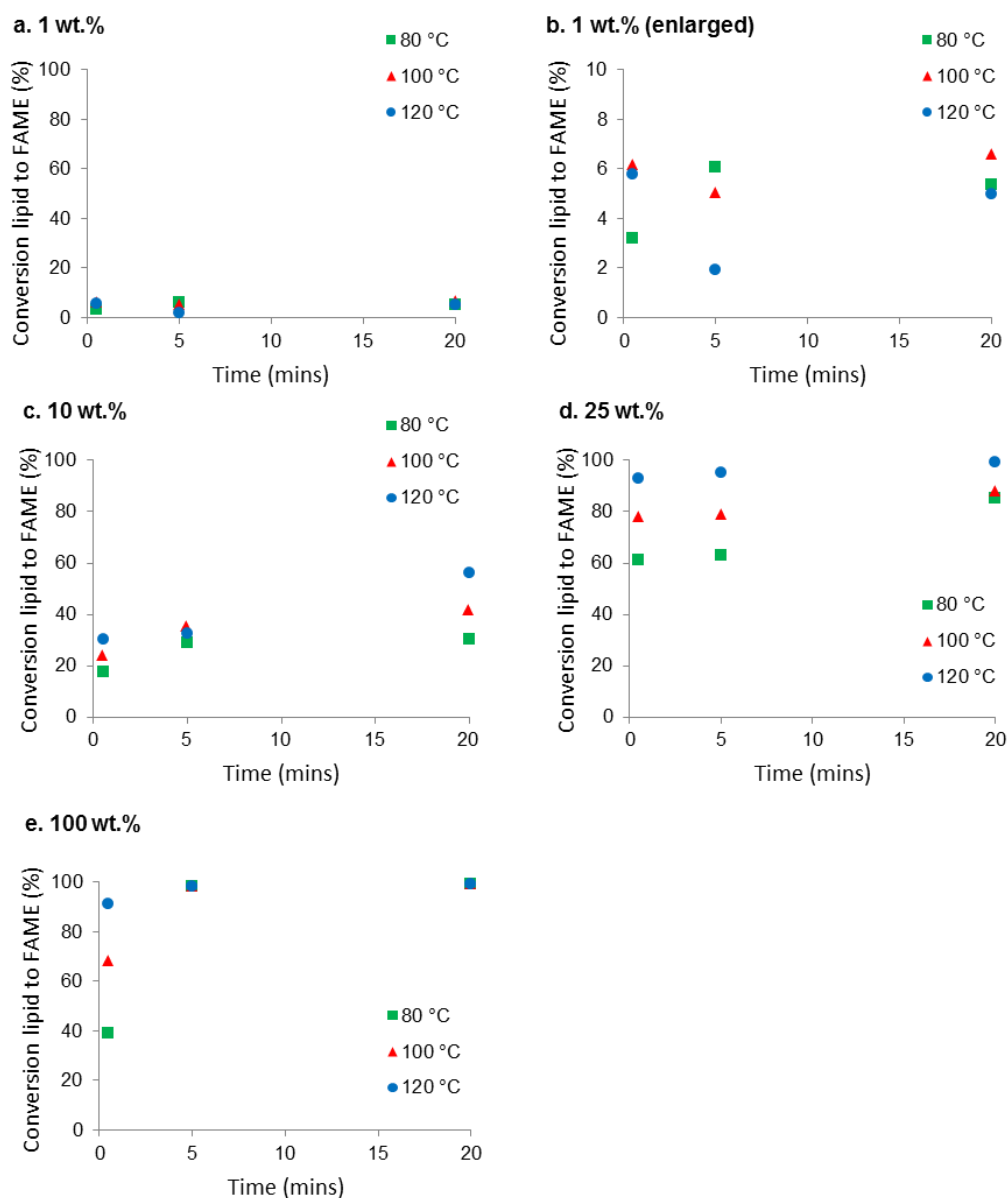


Figure 6.5: Conversion of extracted *Rhodotorula glutinis* lipid to fatty acid methyl ester (FAME) as a function of catalyst (H_2SO_4) loading for the microwave irradiated samples, using Anton Paar monowave 300 reactor. 0.1 g of the freeze-dried *Rhodotorula glutinis* biomass was extracted with 6 ml of 2:1 chloroform:methanol in a 10 ml microwave vial with varying catalyst loadings (1, 10, 25 or 100 wt.%), at three reaction temperatures (80, 100 or 120 °C) and three reaction times (0.5, 5 or 20 min). The pressure and power consumption were measured throughout but not controlled. The FAME was quantified by GC-MS.

6.3.2.2 Lipid yield from microwave extraction

In order to quantify the total lipid yield, all the unreacted lipids from the microwave extractions were transesterified into FAME under reflux conditions, and quantified by GC-MS (Figure 6.6). The yield of lipid obtained via microwave extraction ranged from 24.6% to 34.4% lipid (dry wt.%). A total of 32% lipid was obtained using

Soxhlet extraction after 4 hours, suggesting that under the optimum conditions, microwave extraction could be slightly more effective at extracting the lipid from freeze-dried biomass. This effect has been observed previously with soybean oil, in which 116.3% more lipid was extracted in 1 h compared to 4 h Soxhlet extraction at 60 °C.²⁸⁹ Irrespective of catalyst loading or temperature, the majority of the lipid (~30%) was extracted at 0.5 min reaction time. Increasing the level of catalyst loading had little effect on the total lipid extracted, suggesting that the acid catalyst does not increase the permeability of the yeast cell.

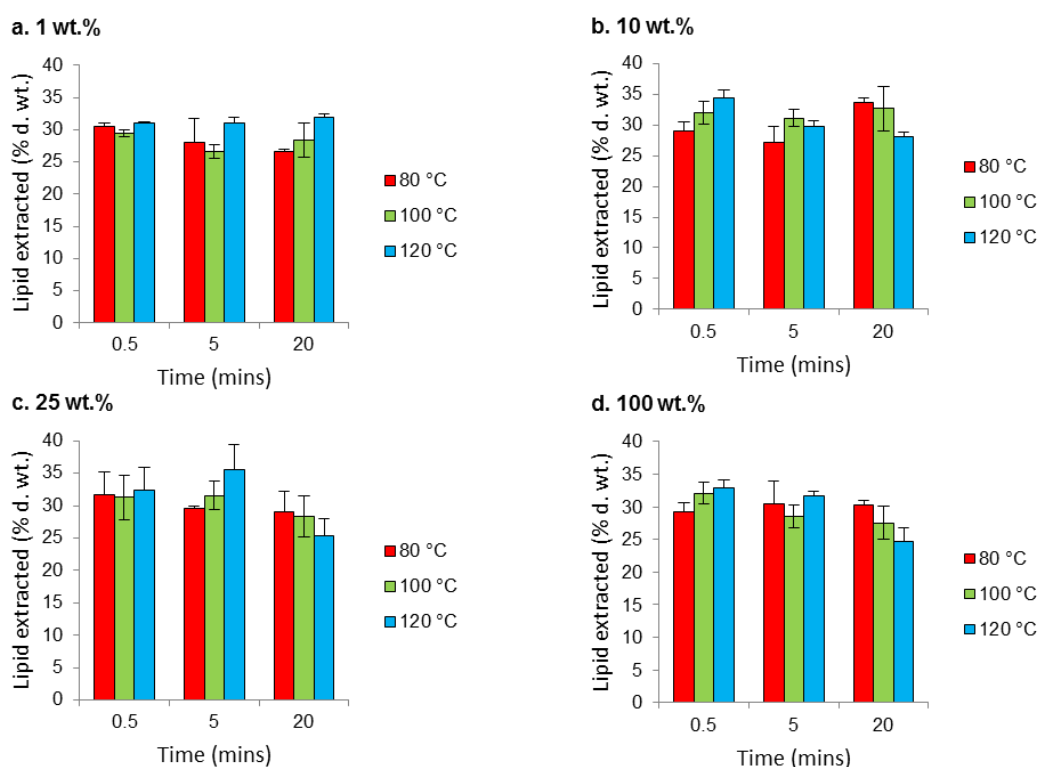


Figure 6.6: The effect of the reaction time (0.5, 5 and 20 min), catalyst loading (1, 10, 25 and 100 wt.%) and temperature (80, 100 and 120 °C) on the total lipid extracted from *Rhodotorula glutinis* biomass using the Anton Paar monowave 30 reactor. Following quantification of the fatty acid methyl ester (FAME) yield, any unreacted oil was transesterified with 0.1 g H₂SO₄ and 2:1 CHCl₃:MeOH under reflux conditions for ≥16 h. The remaining acid and methanol was removed by washing three times with deionised water, before the chloroform was removed under vacuum. The total FAME was quantified by GC-MS analysis.

Once the reaction time was increased to 20 min., the quantity of lipid recovered decreased, especially at higher temperatures. This was particularly exacerbated at a catalyst loading of 25 wt.% and 100 wt.%, suggesting that the harsh conditions may

promote lipid degradation. Longer reaction times have been shown previously to reduce biodiesel yields during the transesterification of waste rapeseed oil using microwave heating.²⁹⁰⁻²⁹¹ The main mechanism of lipid degradation is by oxidation,²⁹² in which polyunsaturated lipids are more susceptible to oxidation than monounsaturated or saturated lipids.¹⁸⁴ Oxidative degradation of lipids is also accelerated at high reaction temperatures.²⁹³ To assess this, the FAME profile from the total lipid extracted was analysed by GC-MS (Figure 6.7).

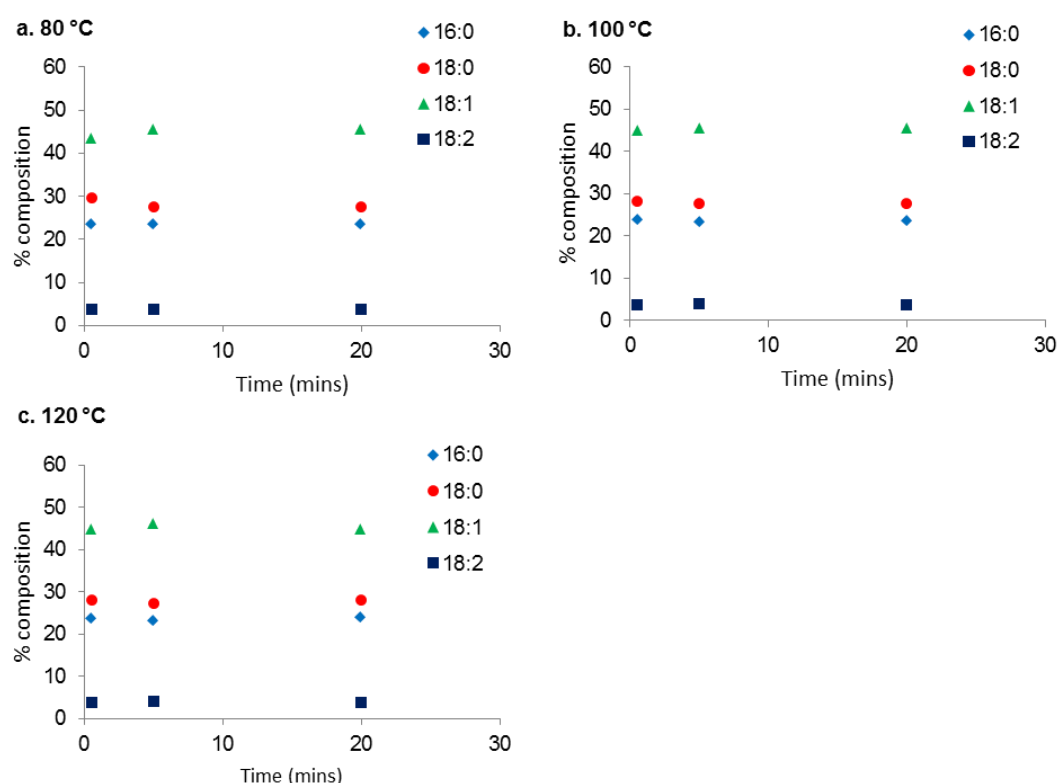


Figure 6.7: Change in the major fatty acid methyl ester (FAME) components on varying reaction time (0.5, 5 and 20 min) and temperature (80, 100 and 120 °C) of the microwave reaction, using the Anton Paar monowave 300 reactor, for *Rhodotorula glutinis* biomass using 100 wt.% H₂SO₄. Any unreacted oil was transesterified with 0.1 g H₂SO₄ and 2:1 CHCl₃:MeOH under reflux conditions for ≥ 16 h. The remaining acid and methanol was removed by washing three times with deionised water, before the chloroform was removed under vacuum. The FAMES were quantified by GC-MS analysis by comparing to known standard FAMES in the C₈ – C₂₄ range.

No change in the FAME profile was observed irrespective of the reaction conditions or the quantity of lipid extracted. Similarly, no products from the oxidative breakdown of fatty acids were observed in the GC-MS chromatograph. This demonstrates that the FAME was not degrading even at high catalyst loading and longer reaction times. The lower lipid yield may therefore be attributable to side

reactions occurring during the breakdown of other cellular components. For example, intracellular water may be released at harsher extraction conditions which could form free fatty acids (FFA). Indeed, some evidence also suggests that water can be produced from the glyceride oils at high power loadings, elevating the production of FFA.¹⁸⁴ The total lipid yield did not however differ considerably from the lipid obtained via Soxhlet extraction, and thus this effect was minor.

6.3.3 Energy return on investment

Using microwave extraction, 30 seconds was a sufficient reaction time to extract the lipid from the cell, though longer reaction times or higher temperatures were required in order to fully transesterify the lipids to FAME. Using higher temperatures and longer reaction times use more energy, and it is important to understand the energy balance of the extraction to assess the feasibility of using microwave irradiation. As such, the energy return on investment (EROI) of the extraction and transesterification step was assessed. The energy used in the microwave extraction was calculated as a percentage of the energy present in the resulting lipid, using the LHV (lower heating value) of the lipid as 39.99 MJ/kg.

Once the calculated value for the microwave energy demand is greater than 100% (indicated by the red dashed lines), more energy is used to extract the lipid than is present in the lipid itself (Figure 6.8). For the extraction at 120 °C and 30 min, the energy demand was 106%. The EROI increased (% decrease) as the reaction temperature and time was decreased. For example, at 80 °C over 0.5 min, there was six times more energy in the extracted lipid than the energy consumed in the extraction. As lipids are readily soluble in chloroform, it is unlikely that the energy input will change substantially as a result of increased cellular lipid. As such, the results of the EROI from the experimental calculations were extrapolated to assess the effect of varying lipid content on the EROI. Unsurprisingly, the EROI is higher at higher lipid yields. However, for the EROI to be greater than zero, the lipid content of the yeast must be greater than 25% (w/w) for an extraction time of 20 min or greater than 10 - 15% for shorter extraction times (Figure 6.8, b & c).

Some oleaginous yeast are capable of producing lipid contents of between 60-70% dry weight of the cell. However, Ratledge *et al.* demonstrated that in order to balance the increasing demand for nutrients and decreased production of biomass, the optimal level of lipid production from heterotrophic organisms is around 40% (w/w).³⁵ This is because, aside from being used for the synthesis of the oil, the substrate is also used to make the cell biomass. As such, higher lipid yields are associated with making more oil, but less oil-free biomass. Even under optimum continuous cultures, the maximum lipid yield reported is 22.4 g oil per 100 g glucose.³⁵ As such, it is biologically and energetically unreasonable to aim for an oil content of greater than 40% (w/w).

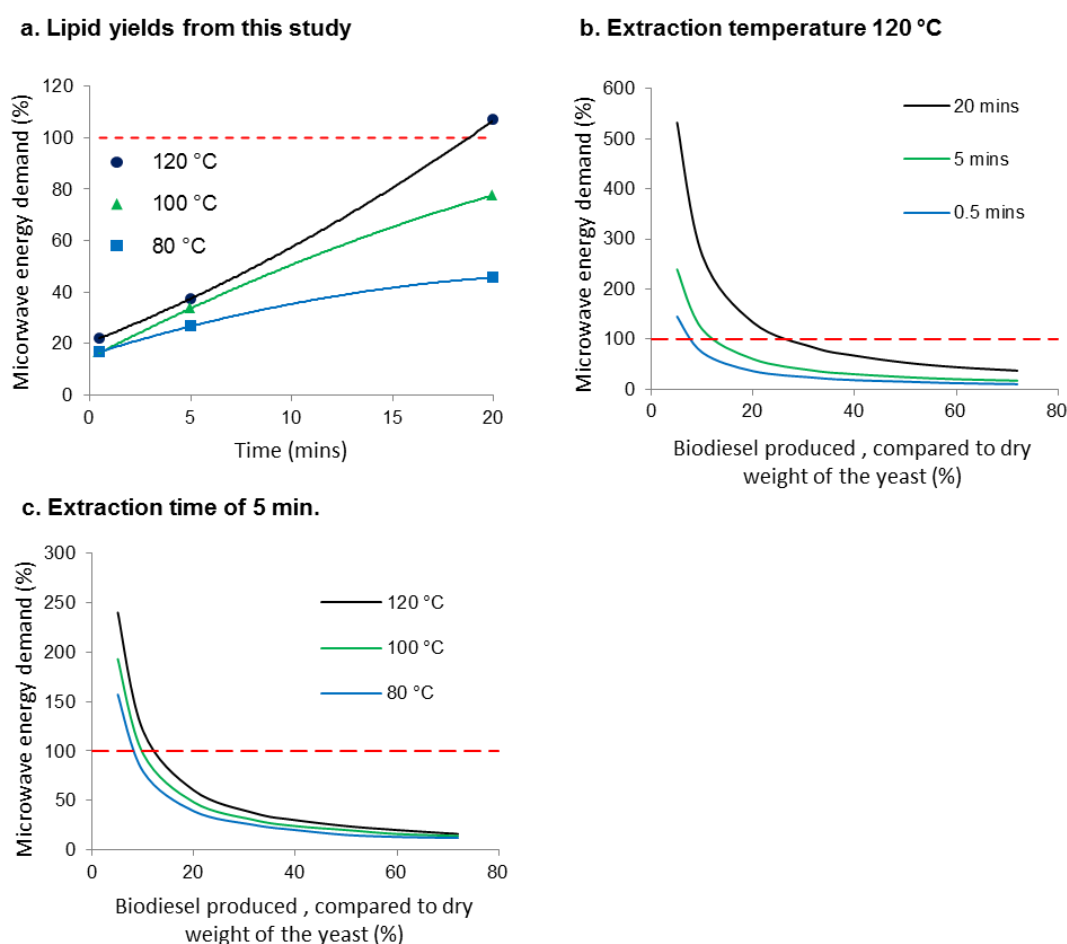


Figure 6.8: Energy return on investment of the microwave extraction of yeast oil using: a. The lipid content found in the *Rhodotorula glutinis* biomass from experimental extraction; b. An extrapolation based on a theoretical variable lipid content at 120 °C; c. An extrapolation based on a variable theoretical lipid content over 5 min. Calculations were made using the lower heating value of microbial lipid of 39.99 MJ/kg.

Similar EROI calculations were also applied to the energy content of the biodiesel produced from the microwave reactions (Figure 6.9). Using the FAME profile acquired by GC-MS analysis, the lower heating value of the biodiesel used for the EROI calculations was 40.12 MJ/kg. Due to the low yields of biodiesel that were obtained using a catalyst loading of 10 wt.%, for most of the reactions the energy used in the extraction reactions was greater than the energy content of the resulting biodiesel (Figure 6.9, c). However, once the catalyst loading was increased to 25 wt.%, at a reaction time of 0.5 min, only 20% of the energy present in the fuel is required to run the microwave system. Increasing the catalyst loading to 100 wt.% did not further improve the EROI. At the 20 minute reaction times, irrespective of the catalyst loading, at least 80% of the energy content of the biodiesel is required for the extraction process. While high biodiesel yields are essential for a high EROI, greater catalyst loadings and higher reaction temperatures are more efficient than longer reaction times. While this may require the use of more acid catalyst, the levels used within this study are consistent with a two-stage biodiesel production process for FFA esterification.²⁹⁴

While there is currently little published work surrounding the energy costs of producing oil from yeast, many studies surrounding the life cycle and economic analysis of algal oils have been conducted. Sills *et al.* assessed 14 publications on the life cycle analysis of algal lipid fuels, and determined that using solvent extraction (hexane) followed by transesterification, would require 54% of the energy present in the resulting biodiesel.³²

While the calculations presented here suggest that this could be reduced to around 20%, the results are only applicable to small-scale extractions, and thus caution should be taken when comparing the results to larger extractions present in the literature or industrial processes. The extraction of oils using a continuous microwave system has however been demonstrated for microalgae.²⁷⁴

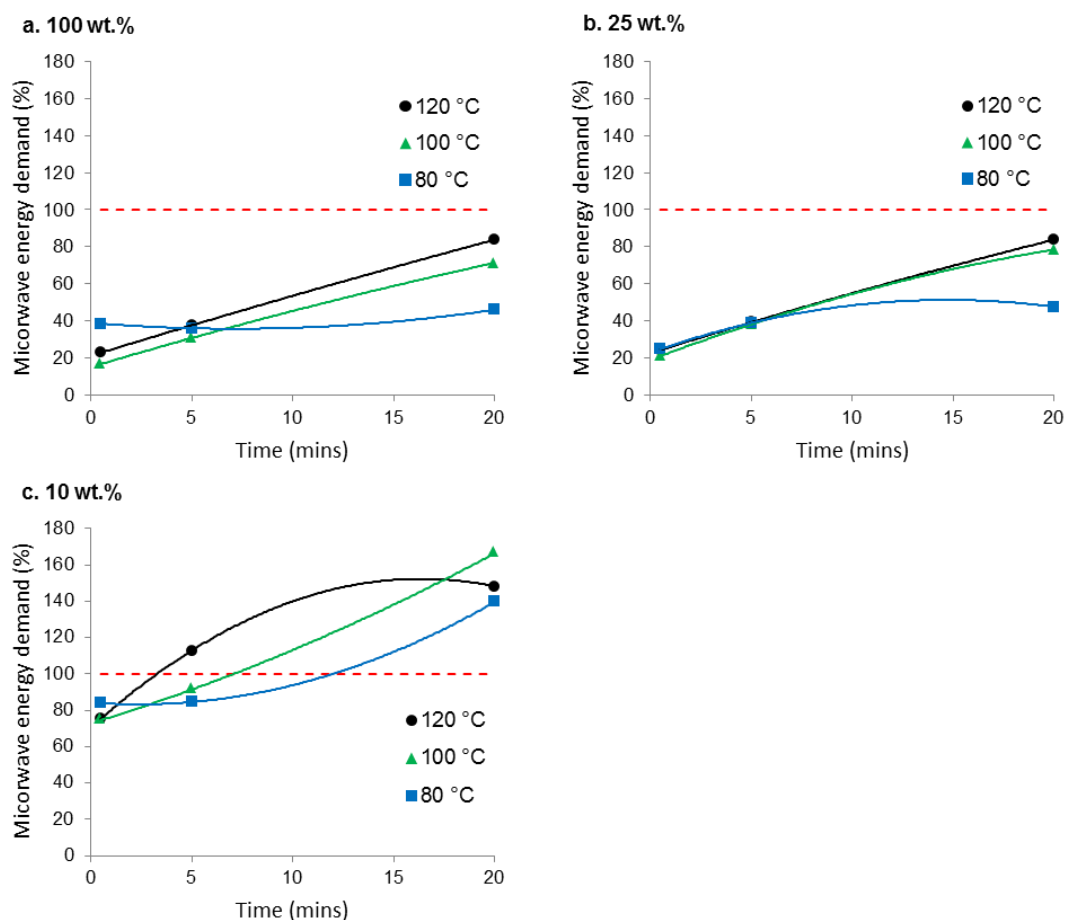


Figure 6.9: Energy return on investment of the microwave extraction for the biodiesel produced using: a. 100 wt.% catalyst; b. 25 wt.% catalyst; c. 10 wt.% catalyst. Calculations used a lower heating value for biodiesel of 40.12 MJ/kg.

Assessing the economics of producing biodiesel from microbial oil using published data, Koutinas *et al.* estimated that the cost of biodiesel using direct transesterification would be 47% higher than indirect transesterification (US \$3.36/L and US \$4.93/L, respectively) due to the high cost involved with direct transesterification.²⁸² The high yielding, short reaction times presented here may help to reduce this cost. Koutinas *et al.* do not however include the cost of the glucose feedstock to achieve a truly representative price analysis. While there are currently no full industrial processes producing biodiesel from yeast oil, DSIR Industrial Development, New Zealand extensively researched the production of a cocoa butter substitute from single cell oils in the 1990s. Reporting on a continual process improvement on the pilot scale, they acknowledged that the economics of SCO production are strongly hampered by the cost of the raw material feedstock.²⁹⁵

This is because lipid production is a relatively inefficient process, requiring five tonnes of hexose sugar to yield one tonne of lipid.³ They proposed that whey lactose offered the only commercially viable substrate that could compete with commodity cocoa butter. Indeed, the valorisation of waste material is a common theme among lipid producers. Neste oil launched Europe's first pilot scale production of microbial oil in Finland in October 2012, producing microbial oil from agricultural and cellulosic residues using oleaginous yeast and fungi.²⁹⁶ During their economic analysis, Koutinas *et al.* calculated that the initial cost of a glucose-rich feedstock should be less than \$0.14/kg. They proposed that food waste, especially carbohydrate-rich products, would be a suitable source for microbial oil production.²⁸² Consequently, the energetics of food waste valorisation to single cell oils comprises the latter half of this chapter.

6.3.4 Energy balance of lipid production from waste food

As more focus is being directed towards the effective management and treatment of waste material, the valorisation of waste resources has gained particular attention. Significant targets were introduced in the 1999 Landfill Directive²²⁷ to reduce the amount of biodegradable waste going to landfill and the Waste Framework Directive²⁹⁷ introduced in 2008 stipulated for stricter waste recycling and energy recovery objectives. With the cost of landfill rising rapidly, the gap between landfill disposal and other technologies is reducing. As described in Chapter 5, food waste valorisation could play a pivotal role in the development of a bioeconomy.²²² Food waste is a complex biomass containing various components such as starch, fats, protein and cellulosic materials and as such, offers many routes for valorisation.²³³ By diverting this organic food waste from landfill and into emerging technologies prevents the uncontrolled generation of methane gases within landfill sites. In 2011, 47% of UK local authorities (LAs) were providing a separate food waste collection service to UK households, and this number is increasing further.²³¹ The treatment of the food waste varies between LAs, but most commonly it is either mixed with garden waste and composted for use as a soil conditioner, or it is transported to anaerobic digestion (AD) facilities.²⁹⁸ AD is still however in commercial infancy, with only three dedicated AD food waste plants

currently operating in the UK.²³³ During anaerobic digestion, the organic waste is biologically degraded by microorganisms in the absence of oxygen and converted into a biogas. The biogas consists of approximately 70% methane,²⁹⁹ which can be used as an energy source.

One of the most widely used environmental assessment tools for comparing alternative technologies is life cycle analysis (LCA). LCA quantifies the transfer of energy, materials and waste streams over the complete supply chain. This differs from a life cycle impact assessment (LCIA) in which the environmental impact of the resource use on ecological and human health are also taken into account. Waste-to-energy processes are often described as a multi-functional process, as they fulfil more than one purpose. In this case, the waste is diverted from landfill and energy is generated in a single process. Using a life cycle analysis approach, Evangelisti *et al.* compared three possible technologies for the organic fraction of municipal waste (landfill, incineration or anaerobic digestion), and they concluded that AD was the best treatment option of organic waste.³⁰⁰ This was mostly due to the high moisture content (74 - 90%) of food waste which hinders the direct combustion or gasification of the biomass, and thus biological transformation is a more desirable route for efficient energy extraction. While the majority of the research surrounding the biological transformation of food waste has focused on the utilisation of anaerobic digestion,^{299,301} there are also a few reports that use food waste as a substrate for bioethanol production.^{224,232,302} Less commonly, the production of single cell oils has also been investigated.^{84,303} However, the energy recovered from these processes has not been compared.

The objective of this study was thus to assess the energy balance of oil produced from the oleaginous yeast *Rhodotorula glutinis* grown using food waste in the UK, compared with methanisation of the same food waste via anaerobic digestion. This work was completed in collaboration with Airbus Innovation Group, Suresnes.

6.3.4.1 Functional unit and boundaries

Different functional units can be used depending on the scope of the study.³⁰⁰ The functional unit is used to describe and quantify the properties of the product, and

provides a reference to which all the inputs and outputs of the system can be calculated.²²³ The function of this study was the production of bioenergy, either as oil or as a biogas. The functional unit for this study was therefore the total amount of energy resulting in the raw biogas or lipid from the food waste in MJ/kg wet food waste. The waste considered in this study was 5000 kg of segregated food waste. Figures 6.10 and 6.11 illustrate the systems for the two alternative waste-to-energy routes considered for SCO production and AD (methanisation), respectively. A gate-to-gate system boundary was used for this study; the transportation of the food waste to the fermentation facilities, scrubbing of the biogas or chemical upgrading of microbial lipid was not taken into consideration for these calculations. Furthermore, waste streams such as the biomass residue following hydrolysis, the oil-free yeast biomass or the resulting anaerobic digestate (liquid) was not taken into account during the energy transfer calculations. These themselves do however have the potential for valorisation.

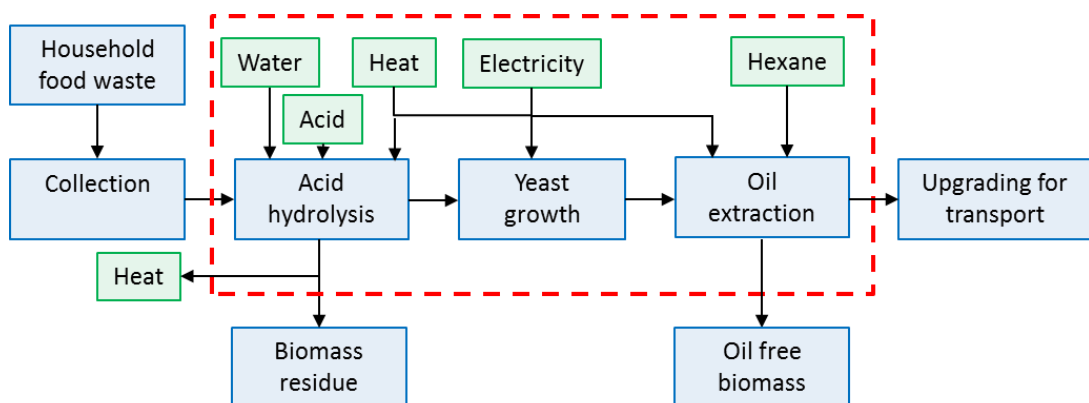


Figure 6.10: Overview of single cell oil production from food waste with oleaginous yeast. The arrows represent material and energy transfer within the system. The red dashed line indicates the system boundary. The blue boxes represent the upgrading of food waste within the system, with the green boxes representing additional material and energy requirements.

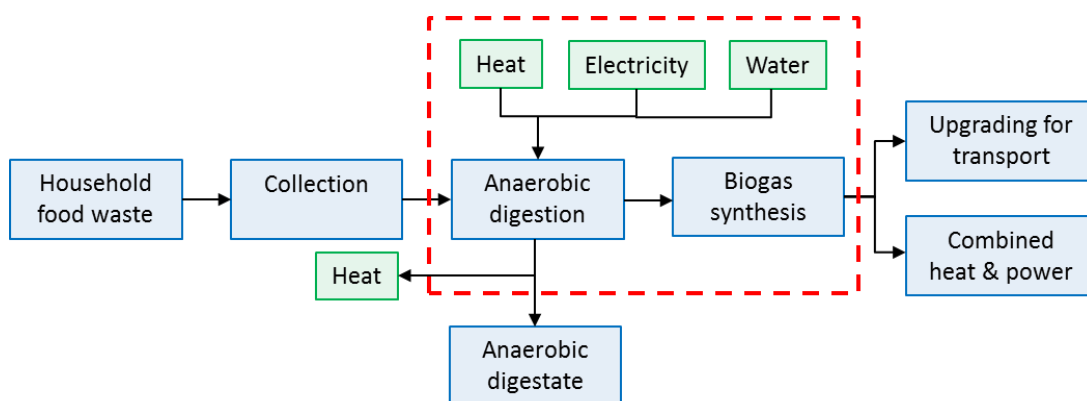


Figure 6.11: Overview of the anaerobic digestion system analysed. The arrows represent material and energy transfer within the system. The red dashed line indicates the system boundary. The blue boxes represent the upgrading of food waste within the system, with the green boxes representing additional material and energy requirements.

6.3.5 Single cell oil (SCO) production process

The production of SCO from food waste is a multi-step process in which the sugars are extracted from the waste biomass, the oleaginous microorganisms are cultivated and the oil extracted. The resulting oil can then be used raw or upgraded through transesterification and/or hydrotreatment to yield a suitable fuel product.

6.3.5.1 Hydrolysis of food waste

Depending on the feedstock source, the actual composition of the food waste can differ significantly (Table 6.1). Variation in the food waste composition strongly affects the yield of oil obtained. For this study, data from Pleissner *et al.* was used (food waste A) due to it having the largest carbohydrate fraction.²³⁹

Table 6.1: Characterisation of food waste within published literature.^{239,303,232,224}

	Pleissner et al. (2014)			Pleissner et al. (2013)		Yan et al. (2011)	Matsakas et al. (2014)
	Food waste A	Food waste B	Bakery waste	Food waste	Bakery waste	Food Waste	HFW
Carbohydrates (mg g ⁻¹)	738.4	470.3	654.5	332.6	620.1	638.7	338.1 [†]
Starch (mg g ⁻¹)	612.3	361.5	316.7	NR	NR	NR	NR
Proteins (mg g ⁻¹)	57.9	99.3	98.2	103.7	42.5	213.4	105.1
Lipid (mg g ⁻¹)	73.8	373.3	265.8	150.3	190.2	124.2	119.1
Cellulose (mg g ⁻¹)	NR	NR	NR	NR	NR	19.8	183.0

[†] Soluble carbohydrate. NR = not reported; HFW = Household food waste.

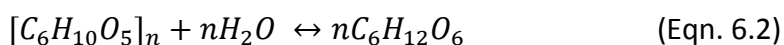
For the conversion of food waste to SCO by oleaginous microorganisms, efficient saccharification of the carbohydrate fraction is required as yeast cannot ferment

starch and cellulose directly. Various types of pre-treatment of the food waste have been reported in the literature, including enzymatic-,^{224,232} whole cell fungal-^{239,303} and thermochemical hydrolysis.²³⁸ In order to reduce the particle size of the food waste, shredding is also commonly used as a pretreatment method prior to hydrolysis. Within this study, thermochemical acid hydrolysis was used, with 1% (w/w) sulphuric acid. As demonstrated in Chapter 4 (Section 4.3.6), thermochemical hydrolysis with sulphuric acid resulted in a higher quantity of soluble sugars compared to enzymatic hydrolysis of food waste (Section 4.3.5), and thus was used for this study. This step also acts as a sterilisation step to prevent microbial contamination within the yeast culture.⁸³ The heat energy required to hydrolyse the food waste was calculated according to Equation 6.1:

$$Q = C_p \cdot m \cdot \Delta T \quad (\text{Eqn 6.1})$$

Where Q is the amount of heat (kJ), C_p is the specific heat capacity (kJ/ kg.K), m is the mass of the wet food waste (5000 kg) and dT is the temperature difference. The calculation was based on the specific heat capacity of water (4.19 kJ/(kg.K))³⁰⁴ and a temperature difference of 80 °C. The input water temperature was assumed to be 20 °C, with a reaction temperature of 100 °C.

Pleissner *et al.* reported that 73.8% of dry food waste is carbohydrate, of which 61.2% is starch.²³⁹ If all the glucose contained within the starch structure were hydrolysed, this would yield 680.14 mg glucose/g dry food waste (Equation 6.2).



$$612.13 \text{ mg} + 68.01 \text{ mg} \leftrightarrow 680.14 \text{ mg}$$

However, yields of approximately 80% are more common.⁸⁴ As such, calculations have been made using a glucose yield of 544.11 mg/g glucose.

Table 6.2 Energy required for the pretreatment and hydrolysis of food waste.

	Input (kg)	Input (MJ)	Output (kg)	Output (MJ)
Wet food waste ^a	5000	19000	-	-
Maceration ^b	-	565.72	-	-
Heat ^c	-	1676	-	-
H ₂ SO ₄	50	-	-	-
Glucose ^d	-	-	544.11	8488.1
Residual biomass ^e (dry)	-	-	455.89	8661.9
Energy loss	-	-	-	4091.7

^aWet food waste = 20% dry food. LHV dry food waste = 19 MJ/kg; LHV glucose = 15.6 MJ/kg.

^bMacerator: 4-shaft shredder. 11kW, throughput 350 kg/h.

^cHeat capacity of water is 4.19 MJ/kg.°C; ΔT is 80°C.

^dGlucose yields are based on 80% hydrolysis of the glucose in the starch fraction (680.14) of the food waste.

^e1000 kg (dry food) – 544.11 (glucose)

6.3.5.2 Yeast cultivation

The yeast was assumed to be cultured in a semi-continuous manner in a continuous stirred tank reactor (CSTR), producing yeast with an oil content of 50% (w/w) of the total dry weight. The energy requirement of the fermenter for the yeast growth (stirring, airing and temperature regulation) was taken to be 3.99 MJ/kg of yeast.³⁰⁵ Nonus *et al.* estimated that 1 – 3 kW/m³ power is required for an industrial bioreactor, and thus this value was based on a 2 kW.m³ industrial reactor, producing 130 kg/m³ of biomass in 73 h in a fed-batch system.³⁰⁶ The maximum theoretical lipid yield on glucose is 0.34 g lipid /g glucose.⁹⁶ Due to other cellular processes requiring glucose, even under ideal conditions for lipid production, the lipid yield on glucose is rarely more than 0.22 g/g.^{22,35} Therefore, it would require 5 kg of glucose to produce 1 kg of lipid in a yeast culture process. From 544.1 kg of glucose (5000 kg hydrolysed wet food waste, 20% d.wt) it would therefore be possible to yield 108.8 kg lipid (Table 6.3). Ratledge *et al.* suggested that 40% lipid (w/w) was the optimum in order to maximise the overall biomass yield.³⁵ As such, this would result in 272.0 kg yeast biomass (108.8 kg oil plus 163.2 kg oil-free biomass). Centrifugation was used to increase the total solids to 20% (w/w). This data was based on that calculated by Bricout,³⁰⁵ using a mean of the data from Bakx *et al.*³⁰⁷ and Diagouraga.³⁰⁸

Table 6.3: Energy required for the cultivation and harvesting of the yeast.

	Input (kg)	Input (MJ)	Output (kg)	Output (MJ)
Glucose	544.11	8488.12	-	-
Electricity ^a	-	1085.50	-	-
Centrifugation ^b	-	56.32	-	-
Yeast biomass (20% solids) ^c	-	-	1360.3	8988.7
Energy loss	-	-	-	641.0

LHV glucose = 15.6 MJ/kg; LHV microbial biomass (dry) is 33.04 MJ/kg.

^a3.99 MJ/kg yeast for the fermentation.; ^b0.207 MJ/kg; ^cYeast biomass is based on a maximum of 40% (w/w), with 100 g glucose yielding 20 g oil and 30 g oil-free biomass, with 20% total solids, 80% water.

6.3.5.3 Oil extraction

The oil was assumed to be extracted from wet biomass with hexane. Energy data for the extraction was adapted from Lardon *et al.*,³⁰⁹ in which the authors calculated that extracting oil from the wet microalgal biomass (*Chlorella vulgaris*) was the more favourable option.³⁰⁹ This process uses direct extraction of the oil from wet biomass in a 1:1 volume ratio of solvent:biomass, assuming an extraction efficiency of 70%. They calculated that for oil extraction of wet biomass, 2.66 MJ/kg was required in heat, 1 MJ/kg yeast in electricity and 6.56 g heat loss /kg yeast.³⁰⁹ Once the solvent has been recycled, two products are yielded: a crude oil and an oil-rich cake.

Table 6.4: Energy required for the extraction of the oil from the yeast biomass.

	Input (kg)	Input (MJ)	Output (kg)	Output (MJ)
Yeast slurry	1360.3	8988.7	-	-
Electricity ^a	-	272.05	-	-
Heat ^b	-	723.67	-	-
Hexane ^c	1360.3	-	8.71	-
Lipid ^d	-	-	76.18	2894.67
Oil cake	-	-	195.87	2049.8
Energy loss	-	-	-	5039.95

^a1MJ/kg dry yeast electricity; ^b2.66 MJ/kg dry yeast heat; ^c6.4 g/kg yeast hexane loss

^dAssumes an extraction efficiency of 70%; ^eOil cake consists of the residual biomass and 30% of the intracellular oil. Residual biomass LHV = 4.96 MJ/kg. LHV microbial biomass is 33.04 MJ/kg;²⁰ LHV of microbial oil is 38 MJ/kg.

6.3.5.4 Overall energy balance for SCO production

The energy transfer through the SCO process is shown in Figure 6.12. Overall, the primary energy input was 0.88 MJ/kg wet food waste. This was comprised of 0.45 MJ/kg, 0.23 MJ/kg and 0.20 MJ/kg for the hydrolysis, oil production and oil extraction, respectively. This indicated that the majority of the energy is involved

during the pretreatment and hydrolysis step, and was mainly due to the energy involved with heating the food waste slurry. Industrial implementation of single cell oil production is largely dependent on the feedstock used,²⁸² and therefore the costs associated with the hydrolysis step would need to be reduced significantly. Assessing only the energy contained within the oil, the energy output was 0.58 MJ/kg food waste, and thus there was a net energy loss of 0.30 MJ/kg. However, only 15.2% of the energy contained within the food waste resulted in the oil. As discussed later, there are many other waste streams within the SCO process that may be suitable for valorisation.

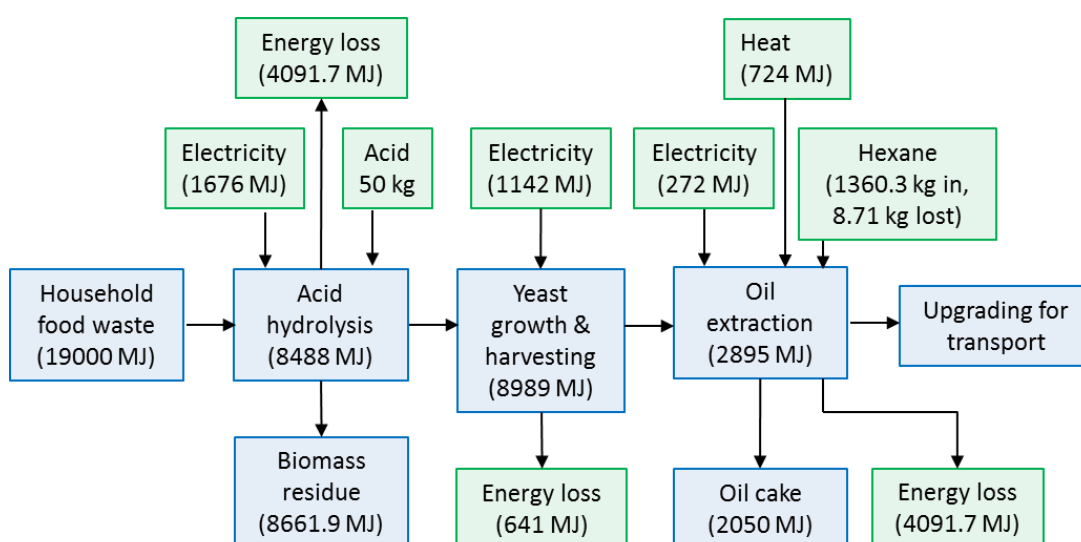


Figure 6.12: Overall energy transfer through the single cell oil process. The numbers in parentheses indicate the energy transferred to the next stage of the flow chart (blue boxes), or energy/material added or lost from the system (green boxes). The functional unit was the energy resulting the raw yeast oil from the food waste in MJ/kg of wet food waste. Calculations were based on the upgrading of 5000 kg of segregated food waste.

6.3.6 Anaerobic digestion

Anaerobic digestion (AD) of food waste is becoming an increasingly popular method as an economic and environmentally effective waste treatment method.²²⁸ Compared to SCO production it is a relatively simple, though biochemically complex process based on a single-stage or two-stage anaerobic digestion process. For all systems, AD occurs in four separate phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis.³⁰¹ In single-stage systems, this occurs simultaneously in a single reactor, whereas in the two-stage process, hydrogen and methane are

generated in separate reactors.⁸³ The system used in this study was based on a single-stage process, which is widely employed for the treatment of municipal solid waste (Figure 6.11).

Depending on the operating conditions of the AD plant (e.g. temperature, retention time and digester type) the energy requirements can vary significantly. Most significantly, dry raw materials such as wheat straw require more pre-treatment than wetter materials. For food waste, mesophilic (~35 °C) AD is the most stable. Thermophilic digestion (~55 °C) enables faster extraction of biogas, but it can be sensitive to input composition when using food waste.³¹⁰ For this assessment, data from Evangelisti *et al.* was used;³⁰⁰ assuming a continuous, single-stage, mixed tank mesophilic reactor operating at 35 °C (primary data from Berglund and Börjesson).³¹¹ This process assumes continuous heating to 35 °C, as well as a sterilisation pre-treatment step of 70°C for one hour prior to digestion.³⁰⁰ Similarly to the energy balance for SCO production, the energy input for AD was calculated based on 5000 kg of food waste (wet). Water was also added to reduce total solids in the mixture to 10% (w/v) dry matter content. This data was based on a large-scale biogas plant, as smaller plants require a higher energy input due to poor insulation and limited use of heat exchangers.³¹¹

For AD, the proportion of volatile solids is particularly important because it represents the organic matter available for the complete conversion into a biogas (the remaining solid being the ash content of the food waste). The total solids (TS) within food waste can range from 14.3 – 24.8% (w/w), with the volatile solids (VS) ranging from 85 – 98.2% (VS/TS).⁸³ As such, food waste with a volatile solid (VS) composition of 90% (VS/TS) and total solids (TS) of 20% was selected. The lower heating value (LHV) of dry food waste can also range from 18.4³¹² – 20.15²²⁸ MJ/kg, and thus the LHV used for this study was 19 MJ/kg.

For 5000 kg of food waste, with a total solid (TS) composition of 20% and a volatile solid composition of 90% (VS/TS), resulted in 900 kg of VS. Using a stoichiometric equation of the substrate ($C_{16.4}H_{29}O_{9.8}N$), Browne & Murphy calculated the theoretical biomethane potential of food waste to be approximately 550 L.CH₄/kg

VS, with a methane content of approximately 57%.²²⁸ Zhang *et al.* achieved a yield of 435 L.CH₄/kg VS with an average VS destruction of 81%.²⁹⁹ This was assuming a total biogas yield of 465.4 m³/tonne of dry material, with a CH₄ and CO₂ content of 73% and 27%, respectively.²⁹⁹ Given that this latter data is experimentally derived, this lower yield of methane was used for the energy balance. As such, 391,500 L of methane was calculated to be generated from 5000 kg of wet food waste (Table 6.5). At a density of 0.71 g/L²²⁸ and a LHV of 50 MJ/kg, 13,898.5 MJ of energy would be contained within the methane gas.

Table 6.5: Energy and material transfer for the production of methane from food waste by anaerobic digestion.

	Input (kg)	Input (MJ)	Output (kg)	Output (MJ)
Food waste (wet) ^a	5000	19000	-	-
Electricity ^b	-	1150	-	-
Heat added ^b	-	1600	-	-
Water to decrease to 10% solids ^c	5000	165	-	-
Methane yield ^d	-	-	258.4	13898.5
Carbon dioxide yield	-	-	286.7	-
Remaining biomass (d.wt) ^e	-	-	454.9	-

^aLHV of dry food waste was 19 MJ/kg.

^bData refers to a single-stage, continuous stirred tank reactor operating at 35°C with a 10% dry matter content, using sorted municipal organic waste.

^c33 MJ/tonne additional energy when the total solids of the input steam is >10%.³¹¹

^dMethane yield is assumed to be 435 L.CH₄/kg VS.

^eUnconverted biomass and biomass of anaerobic microorganisms.

These calculations indicated that the primary energy input corresponds to 20.8% of the energy content of the biogas produced, with an overall energy yield of 2.77 MJ/kg wet food waste. This is analogous to Berglund & Böejesson, who suggested a primary energy input of 20-40% of the resulting biogas.³¹¹ This can be as high as 50% depending on the feedstock. The lowest energy inputs are derived from food industry waste without gas upgrading, which is comparable to the results presented in this study.³¹¹ If the biogas were to be used as a transportation fuel or heating fuel, upgrading would need to occur to remove unwanted gases such as CO₂, followed by pressurisation. This process is estimated to be approximately 5% of the energy content of the resulting biogas.³¹¹ Once transportation of the food waste to the digestion facilities and upgrading/inefficiencies surrounding CHP are taken into account, this figure would increase. Methane losses during the digestion process,

storage of the digestate and upgrading are also possible. These have previously been determined as 2-3% of the total methane produced.³¹¹ Methane has a global warming potential of 23 times that of CO₂ over a 100 year period, and as such these losses are not insignificant.²²⁸

In addition to the biogas, a nutrient-rich digestate is also produced during the AD process which can be used as a fertiliser or soil conditioner.⁸³ This is because the concentration of macro- and micro-nutrients present in food waste do not change significantly during AD and would therefore provide the essential elements for plant growth if used as an organic fertiliser.²⁹⁹ However, the Animal by-products regulation existing in EU countries only allows source-segregated digestate (food waste not mixed with other food waste or other waste material) to be spread on agricultural land, which should be taken into account when attributing a use to this nutrient-rich stream.²²⁸

6.3.7 Comparison of SCO and AD processes

The primary energy input for the SCO process was calculated to be 0.88 MJ/kg wet food waste, with an energy yield in the oil to be 0.58 MJ/kg. For the process analysed, this indicated an energy loss of 0.30 MJ/kg food waste. In contrast, the primary energy input for the AD process was calculated to be 0.58 MJ/kg wet food waste, with an energy yield of 2.77 MJ/kg, thus an energy surplus of +2.19 MJ/kg food waste. However, when using a life cycle approach to compare processes, there are always associated uncertainties, especially when the data is not gathered at the site being assessed. Therefore caution should be used when drawing on the conclusions found. This being said, the main area of energy input for the SCO process was during the hydrolysis of the food waste. To decrease this energy input, enzymatic hydrolysis operating at lower temperatures may be preferable. However the high cost associated with large-scale enzyme loading may be detrimental to the economic viability. Furthermore, high glucose concentrations can result in catabolic repression of the enzymes.⁸³ Simultaneous saccharification and fermentation (SSF) has therefore been reported as an alternative in which the enzyme hydrolysis and subsequent fermentation occur simultaneously, thus mitigating the risks of allosteric repression and reducing processing costs.⁸³ Kim *et al.* explored SSF for the

production of bioethanol from food waste, and found that ethanol yields reduced by 28% compared to separated hydrolysis and fermentation.³⁰² However, energetically and economically this may be a preferred method for SCO production.

The biogas derived from AD can be used for heating, electricity generation, as a vehicle fuel or distributed onto the natural gas grid.³¹¹ In contrast, the oil obtained from the SCO process can be used to produce road transport fuel, aviation fuel, or as a platform commodity for upgrading into materials such as plastics.²²² The retail price of *first generation* biodiesel is approximately US \$1.13 /kg (assuming a density of 0.88 kg/L), however oil prices can reach as high as US \$45-50 /kg for plant-derived lipids that are high in linolenic acid (18:3).²⁸² In comparison, the retail price of methane, has a retail price of US \$0.60 /kg (assuming a density of 0.66 kg/m³), the price of which will be prone to fluctuation if more AD facilities and hydraulic fracturing sites are commissioned.³¹³

During the SCO process, only 15% of the energy content of the food waste resulted in the final oil. This was mostly due to only some of the carbohydrate in the food waste being converted into oil, with the protein, oil and unconverted lignocellulose fraction of food waste unconsumed by the reaction. Silva *et al.* found that more biogas was produced when used cooking oil and cooked meats were added to the reactor,³¹⁴ and thus the unconverted products of the SCO process may make an ideal substrate for the AD process. Indeed, it would also be a valorisation route for the spent yeast biomass following oil extraction. An integrated SCO and AD process is therefore proposed in Figure 6.60, in which the carbohydrates within the food waste are transformed into oil with the remaining biomass converted into a methane-rich biogas. Depending on the lipid profile of the oil produced, this could be transesterified or hydrogenated to yield a road transport and aviation fuel, respectively, or be a suitable replacement for palm oil as discussed in detail in Chapter 3 (Section 3.1.3).

Due to the large number of different species of bacteria associated with AD processes, it can make processes difficult to compare and have a complete understanding of how the different species interact with one another, especially

with variable feedstocks. This is especially true for the proposed integrated process in which a fraction of the food waste is being removed prior to anaerobic digestion. For complicated waste streams such as food waste these problems are exacerbated due to the many biochemical pathways occurring simultaneously during the breakdown of different substrates.³¹⁰ Therefore greater exploration into the potential of a coupled SCO and AD process from food waste is required.

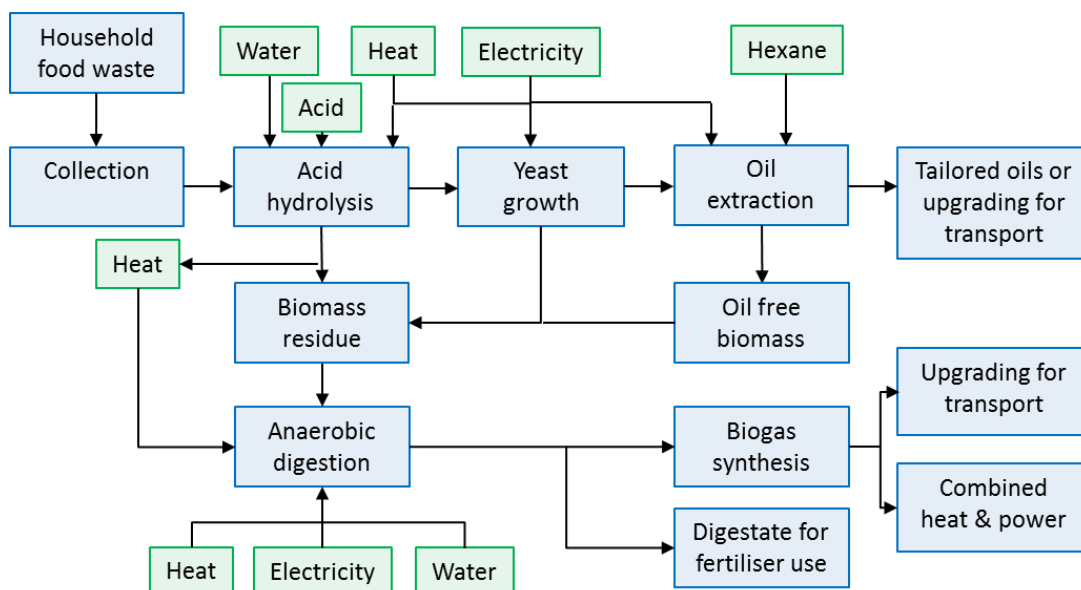


Figure 6.13: A proposed integrated system for single cell oil and biogas production from food waste. The arrows represent material and energy transfer within the system. The blue boxes represent the transfer of food waste within the system, with the green boxes representing additional material and energy requirements.

6.4 Conclusion

The work presented here demonstrated that microwave irradiation is a suitable method for the simultaneous extraction and transesterification of lipids from *R. glutinis*. The same yield of oil was obtained in 30 s at 120 °C comparable to 4 h of Soxhlet extraction. However, in order to fully transesterify the resulting oil, 25 wt.% catalyst loading was required at longer reaction times. When assessing the energy return on investment (EROI), long reaction times were found to be most detrimental to the EROI. Low levels of catalyst loading, also negatively affected the EROI. As such, providing the system is optimised to the feedstock used, microwave irradiation was shown to offer a powerful technique for laboratory use as well as to reduce future industrialised biofuel processes.

The latter half of this chapter assessed two potential value-added routes are though the production of single cell oils (SCOs) from oleaginous microorganisms, or by methanisation via anaerobic digestion by comparing these two routes energetically. As SCOs only make use of the carbohydrate fraction of food waste, and the process involves an energy-demanding pretreatment step, there was a net energy loss across the system. In contrast, anaerobic digestion produced an energy surplus, but methane offers limited end uses. Therefore, an integrated system was proposed which offers the potential for a high-value oil product, with the energy most likely derived from combined heat and power though the combustion of methane produced by anaerobic digestion.

CHAPTER 7:

CONCLUSIONS, GENERAL DISCUSSION & FUTURE DIRECTION

“Don’t cry because it’s over, smile because it happened.”

Dr. Seuss

7.1 Conclusions

The approaches and results presented in this thesis represent a multidisciplinary perspective on some of the challenges associated with producing a commercially-viable yeast oil for use if fuel and commodity applications. The key conclusions from this research are presented below, alongside the initial aims of the project (in bold) and how the aims were achieved:

1. Develop understanding of the effect of environmental growth conditions on the lipid productivity from the yeast, *Rhodotorula glutinis* and *Rhodotorula minuta*.

The use of experimental design, incorporating a response surface methodology examined the effects of varying C/N ratio and temperature on the lipid yield and lipid profile of the resulting oil. The resulting oil was compared to fuel and palm oil specifications.

- The oil from *Rhodotorula glutinis* was shown to be highly variable depending on the C/N ratio and growth temperature, but the response surface methodology was highly successful in indicating growth conditions suitable for bespoke lipids with tailored properties. Following transesterification, the conditions yielded an oil high in oleic acid resulting in a biodiesel comparable to rapeseed methyl ester, whereas the highly saturated oil had similar properties to palm oil.
- The oil obtained from *Rhodotorula minuta* was similar regardless of the C/N ratio, but greater biomass and lipid were produced at lower cultivation temperatures. The lipid profile suggested that upon transesterification, the resulting biodiesel would make an ideal replacement for petrodiesel.

2. Understand the potential of ultra-sonication to enhance the conversion of glucose to oil using *Rhodotoula sp.* The development of an experimental rig in which a sonication flow-cell was connected to a bioreactor, enabled preliminary investigations into the effect of ultrasonication on *Rhodotorula sp.* at different stages of the growth cycle.

- The use of ultra-sonication during the fermentation process marginally improved glucose-to-oil conversion when used in the stationary phase of

cell growth. No positive effect was shown when ultra-sonication was used during the stationary phase of growth. Further investigation is required to assess if the use of ultra-sonication for SCO production would be viable for commercial processed.

3. Assess the potential for *Rhodotorula sp.* to produce oil from low-cost waste substrates. *Rhodotorula sp.* was grown on model growth media, representing different challenges in the use of depolymerised lignocellulose was constructed (mixed sugar substrates and the presence of microbial inhibitors). Food waste and *Miscanthus giganteus* were also depolymerised and *Rhodotorula sp.* grown on the resulting hydrolysate.

- *R. glutinis* and *R. minuta* were shown to be able to assimilate a wide variety of sugar substrates and showed tolerance to common non-acidic microbial inhibitors present in depolymerised feedstocks. Whereas *R. glutinis* could tolerate low concentrations of acids, *R. minuta* showed little growth in the presence of acids.
- Depolymerised *Miscanthus giganteus* was not deemed a suitable feedstock for the production of oil from *R. minuta* due to low biomass and lipid yields, whereas thermochemically-treated food waste offered greater potential as a substrate for SCO production.

4. Examine the energetics surrounding the extraction of yeast oil using microwave extraction. The use of energy return on investment calculations enabled the experimental data from the simultaneous extraction and transesterification of yeast oil using microwave extraction to be assessed for viability. The experimental conditions tested included varying the reaction time, temperature and catalyst loading.

- Simultaneous extraction and transesterification of yeast oil using microwave extraction was shown to be highly effective under a variety of conditions. Only short reaction times (0.5 min) and sufficient catalyst loading (25 wt.%) yielded viable conditions on the basis of energy return on investment

calculations, providing the oil content of the cell was greater than 20% (w/w).

5. Compare the energy balances for the valorisation of food waste to microbial oil, to the methanisation of food waste using anaerobic digestion. Energy balances using an LCA approach were constructed for both routes using data from the literature.

- Theoretical energy balances undertaken on the valorisation of food waste indicated that there was a net loss of energy across the system in the waste-to-oil route, whereas the waste-to-biogas route yielded a net surplus of energy. Due to the current need for liquid fuel in transportation networks, a combined process to produce oil and biogas was proposed.

7.2 General discussion

Throughout this thesis, the potential of *Rhodotorula sp.*, for the production of sustainable lipids has been demonstrated. It has become clear that both *R. glutinis* and *R. minuta* offer great potential as a more sustainable replacement for petrodiesel than the current production method utilising rapeseed oil, which is directly food-competitive. It has also been demonstrated that under suitable growth conditions, oil from *R. glutinis* would also be a suitable replacement for palm oil (Chapter 3). However, in order for such processes to be economically viable with current technology, researchers need to be savvy, assessing the process as a whole. This notion formed the reasoning for Chapter 4, in which the novel process of sonication was tested for its feasibility to increase glucose conversion efficiencies for SCO production. Although an increase of 24% was observed in the lipid coefficient between the sonicated and non-sonicated fermentation, the culture did not yield more lipid overall due to a lower lipid content of the cells. However, using the analogy of a dose response curve, the conditions tested were only one of several possibilities, and as such further research into this field may prove fruitful.

While *R. glutinis* is known to yield up to 72% lipid with maximum a biomass yield of 185 g/L,⁷⁴ the conditions employed to achieve such claims are likely to be at odds with process engineering and basic economics. This is especially true when developing a production process that is directly competitive with commodity plant oils, whose prices are associated with significant fluctuations, thereby making process development more difficult. Indeed, this was the case for the cocoa butter substitute, produced from *C. curvata*, in which the price of cocoa butter decreased by 40% during the product development process.³⁵

While there was a significant drop in vegetable oil prices during 2009-10, the general market trend is upwards (Figure 7.1). If this projection is reliable, then by 2020 the average price of vegetable oil could be more than US \$2 /kg oil. Koutinas *et al.* estimated that the cost of microbial oil to be US \$3.4 /kg in 2014, suggesting that a 50% reduction in the cost of manufacture is required over the next decade for microbial oils to become cost-competitive with vegetable oils.²⁸² The authors propose that lower costs of feedstocks, adaptation of robust yeast strains, innovative fermentation design and valorisation of by-products are imperative to reduce production costs.²⁸²

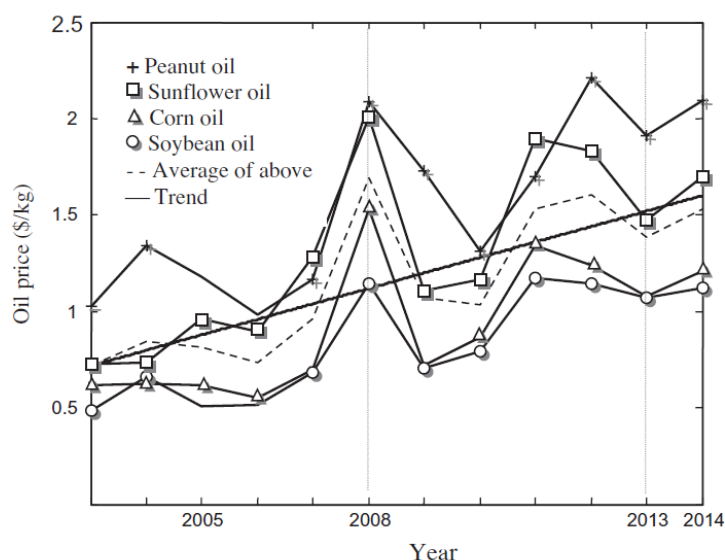


Figure 7.1: Price of common vegetable oils in the U.S. for the period 2003 – 2013, with 2014 values based on data from the U.S. Department of Agriculture.²⁸²

In-line with this, two low-cost feedstocks were tested for their potential substrates for the cultivation of *Rhodotorula sp.* (Chapter 5). Although the growth of *R. minuta* on *Miscanthus* hydrolysate was relatively unsuccessful, producing both low biomass and lipid yields, growth of *Rhodotorula sp.* on depolymerised food waste offered greater promise. Using a thermochemical hydrolysis method, the food waste hydrolysate contained a total sugar concentration of 66 g/L, and yielded biomass concentrations of approximately 10 g/L, with an oleic acid content of 65% (w/w). The development of food waste as a substrate for SCO production will therefore form the basis of the future work section.

Chapter 6 concentrated on the energetics of the production process that formed the basis of this thesis. The first half of the chapter involved the exploration of a one-step system for the extraction and transesterification of yeast oils. Although microwave extraction proved a powerful technique for fast extraction oils, achieving similar yield in 30 sec compared to 4 h of traditional Soxhlet extraction, it is difficult to see how, from a process development perspective this could be used at scale. To the author's knowledge, only a single article has been published describing continuous microwave extraction. This was performed on the microalga, *Scenedesmus obliquus*, of which 77% of the recoverable oil was extracted in 30 min. Energy requirements and process scale-up of this method is yet to be established. Therefore, the results obtained with this thesis, for the time being at least, are only applicable to similar lab-scale studies.

The latter half of Chapter 6 involved the assessment of SCO production from a life cycle analysis perspective in comparison with anaerobic digestion. Due to the large amounts of energy involved during the thermochemical pretreatment of the food waste, SCO production was deemed unfeasible from an energetic perspective. However, the broad range of applications surrounding the use of lipids as a chemical feedstock far outweighs that of methane. As such, a combined production process was proposed in which the methane produced from the anaerobic digestion of residual food waste (after SCO production) could be used in a combined heat and power plant to provide the energy for SCO production.

Another method of increasing the process economics is based on the biorefinery model of producing multiple product streams from a single feedstock. These may include other oleochemical products, such as nutritional polyunsaturated fatty acids, solvents, adhesives, lubricants, plastics or platform chemicals. These products, with a greater profit margin may become economically viable before fuel production does. By products such as pigments could also help with economic viability. Similarly, speciality or substitute oils such as γ -linolenic acid may have to be the focus of SCO production until the cost of production decreases significantly to make the production of commodity oils economically viable. However, as few yeast produce this type of oil in greater quantities than ~1%, significant research into genetic modification would be required, again increasing the costs of manufacture.

7.3 Future direction

With the global population set to reach 9.5 billion by 2075,²²⁹ not only will the production of food need to increase, but the effective management and treatment of biodegradable waste will be of increasing importance. One can look at this scenario in one of two ways. Firstly, with an increasing population, there is the argument that a greater quantity of food waste will be produced. Alternatively, it is equally possible to imagine a scenario that with an increasing demand for food, most likely coupled to increased prices, food will be more highly valued and thus a lower quantity culminates in the waste processing stream. Understanding the change in trends is going to be key in assessing the true feasibility of food waste as a resource for SCO production. One of the biggest challenges surrounding food waste valorisation is the inherent heterogeneity, as it is highly affected by the source from which the waste is derived.²²⁴ Nutritional habits, seasonality and location (both geographically and building type e.g. schools, prisons etc) could affect the composition of the food waste.

Within the UK, household food waste collected at the kerb-side can be contained within newspaper or cornstarch caddy liners. No research has investigated how the presence of these, as well as other contaminants such as the bioplastics, poly lactic

acid (PLA) or polyhydroxybutyrate (PHB) would affect the production of SCO from food waste. Indeed, it would be an ideal scenario to imagine the food waste, as well as the packaging in which it was contained to be able to be treated in the same waste stream.

Furthermore, dietary habits globally are likely to change over the next few decades. Meat consumption has doubled in the past 30 years, and it is expected to double again by 2050. This is partly due to the rising proportion of middle class citizens in China, India and Russia.³¹⁵ However, the production of animal protein is deemed unsustainable due to land usage, GHG emissions and water consumption. The production and consumption of high-protein alternatives is therefore likely to increase. This may include texturised plant protein such as tofu, produced from soya or Quorn™, contain mycoprotein produced from *Fusarium venenatum*. Similarly, Beyond Meat® using soy and pea protein within their products.³¹⁶ More unusual sources of protein may also enter our diets, such as Exo cricket protein bars made from ground crickets. Dried crickets contain 69% protein, and are estimated to be 20 times more efficient as a source of protein than cattle.³¹⁷ Indeed biotechnology may also offer some interesting alternative protein sources. AquaBounty Technologies have been genetically modified Atlantic salmon (*Salmo salar*) through the addition of two genes, to produce the AquAdvantage® salmon, in which a Chinook salmon (*Oncorhynchus tshawytscha*) growth hormone has been inserted under the control of an ocean pout (*Zoarces americanus*) promotor. Instead of the salmon promotor gene which is only active during Spring and Summer, the ocean pout promotor is active all year round which enables the salmon to be reared to full size in 18 months rather than 36 months.³¹⁸ Similarly, the lab-grown burger produced by Prof. Mark Post's research team at Maastricht University may provide an insight as to how we can continue to maintain a red-meat-rich lifestyle without the environmental price tag.³¹⁵

8. References

1. Thevenieau, F.; Nicaud, J.-M., Microorganisms as sources of oils. *OCL* **2013**, *20* (6), D603.
2. Guay, C.; Madiraju, S. R. M.; Aumais, A.; Joly, E.; Prentki, M., A role for ATP-citrate lyase, malic enzyme, and pyruvate/citrate cycling in glucose-induced insulin secretion. *Journal of Biological Chemistry* **2007**, *282* (49), 35657-35665.
3. Ratledge, C., Microbial Lipids: Commercial Realities or Academic Curiosities. In *Industrial Applications of Single Cell Oils*, AOCS Publishing: 1992.
4. Knothe, G.; Krahl, J.; Van Gerpen, J. H., *The Biodiesel Handbook*. AOCS Press: 2010.
5. Ahmed, N., Iraq invasion was about oil. *Guardian* 20th March 2014, 2014.
6. Stephens, E.; Ross, I. L.; Mussnug, J. H.; Wagner, L. D.; Borowitzka, M. A.; Posten, C.; Kruse, O.; Hankamer, B., Future prospects of microalgal biofuel production systems. *Trends in Plant Science* **2010**, *15* (10), 554-564.
7. Sharma, Y. C.; Singh, B.; Korstad, J., A critical review on recent methods used for economically viable and eco-friendly development of microalgae as a potential feedstock for synthesis of biodiesel. *Green Chemistry* **2011**, *13* (11), 2993-3006.
8. Kamm, B.; Kamm, M.; Gruber, P. R.; Kromus, S., Biorefinery Systems – An Overview. In *Biorefineries-Industrial Processes and Products*, Wiley-VCH Verlag GmbH: 2008; pp 1-40.
9. Ragauskas, A. J.; Williams, C. K.; Davison, B. H.; Britovsek, G.; Cairney, J.; Eckert, C. A.; Frederick, W. J.; Hallett, J. P.; Leak, D. J.; Liotta, C. L.; Mielenz, J. R.; Murphy, R.; Templer, R.; Tschaplinski, T., The Path Forward for Biofuels and Biomaterials. *Science* **2006**, *311* (5760), 484-489.
10. Comission, E., National Renewable Energy Action Plan for the United Kingdom. 2009.
11. NNFFC Bio-based Industries: using renewable natural resources and innovative technologies for greener everyday products. http://www.nnfcc.co.uk/news/eu-and-industry-partners-launch-20ac3.7-billion-investments-in-the-renewable-bio-based-economy?utm_source=NNFFC+Mailing+List+2012&utm_campaign=269b9634ab-%E2%82%AC3.7+Billion+Investments+in+Renewables&utm_medium=email&utm_term=0_8e34a0de3f-269b9634ab-228972265 (accessed 5th August 2014).
12. Dean, B.; Dodge, T.; Valle, F.; Chotani, G., Development of Biorefineries–Technical and Economic Considerations. In *Biorefineries-Industrial Processes and Products*, Wiley-VCH Verlag GmbH: 2008; pp 67-83.
13. Biermann, U.; Friedt, W.; Lang, S.; Lühs, W.; Machmüller, G.; Metzger, U. O.; Gen. Klaas, M. R.; Schäfer, H. J.; Schneider, M. P., New Syntheses with Oils and Fats as Renewable Raw Materials for the Chemical Industry. In *Biorefineries-Industrial Processes and Products*, Wiley-VCH Verlag GmbH: 2008; pp 253-289.
14. Donot, F.; Fontana, A.; Baccou, J. C.; Strub, C.; Schorr-Galindo, S., Single cell oils (SCOs) from oleaginous yeasts and moulds: Production and genetics. *Biomass and Bioenergy* **2014**, *68* (0), 135-150.
15. Haas, M. J.; Wagner, K., Simplifying biodiesel production: The direct or in situ transesterification of algal biomass. *Eur. J. Lipid Sci. Technol.* **2011**, *113* (10), 1219-1229.
16. Hoekman, S. K.; Broch, A.; Robbins, C.; Cenicerros, E.; Natarajan, M., Review of biodiesel composition, properties, and specifications. *Renew. Sust. Energ. Rev.* **2012**, *16* (1), 143-169.
17. Papanikolaou, S.; Aggelis, G., Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. *Eur. J. Lipid Sci. Technol.* **2011**, *113* (8), 1031-1051.
18. Karmakar, A.; Karmakar, S.; Mukherjee, S., Properties of various plants and animals feedstocks for biodiesel production. *Bioresource Technol* **2010**, *101* (19), 7201-7210.
19. Islam, M. R.; Beg, M. D. H.; Jamari, S. S., Development of Vegetable-Oil-Based Polymers. *J Appl Polym Sci* **2014**, *131* (18).
20. Meng, X.; Yang, J.; Xu, X.; Zhang, L.; Nie, Q.; Xian, M., Biodiesel production from oleaginous microorganisms. *Renewable Energy* **2009**, *34* (1), 1-5.
21. Sitepu, I. R.; Garay, L. A.; Sestric, R.; Levin, D.; Block, D. E.; Bruce German, J.; Boundy-Mills, K. L., Oleaginous yeasts for biodiesel: Current and future trends in biology and production. *Biotechnology Advances* **2014**, *32* (7), 1336-1360.

22. Ratledge, C.; Wynn, J. P., The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. In *Advances in Applied Microbiology*, Vol 51, Laskin, A. I.; Bennett, J. W.; Gadd, G. M., Eds. 2002; Vol. 51, pp 1-51.
23. Subramaniam, R.; Dufreche, S.; Zappi, M.; Bajpai, R., Microbial lipids from renewable resources: production and characterization. *Journal of Industrial Microbiology & Biotechnology* **2010**, 37 (12), 1271-1287.
24. Chisti, Y., Biodiesel from microalgae. *Biotechnology Advances* **2007**, 25 (3), 294-306.
25. Singh, A.; Olsen, S. I., A critical review of biochemical conversion, sustainability and life cycle assessment of algal biofuels. *Appl. Energy* **2011**, 88 (10), 3548-3555.
26. Mata, T. M.; Martins, A. A.; Caetano, N. S., Microalgae for biodiesel production and other applications: A review. *Renew. Sust. Energ. Rev.* **2010**, 14 (1), 217-232.
27. (a) Koberg, M., Gedanken, A., Optimization of bio-diesel production from oils, cooking oils, microalgae, and castor and jatropha seeds: probing various heating sources and catalysts. *Energy & Environmental Science* **2012**, 5 (6), 7460-7469; (b) Lam, M. K.; Lee, K. T., Microalgae biofuels: A critical review of issues, problems and the way forward. *Biotechnology Advances* **2012**, 30 (3), 673-690.
28. Amaro, H. M.; Guedes, A. C.; Malcata, F. X., Advances and perspectives in using microalgae to produce biodiesel. *Appl. Energy* **2011**, 88 (10), 3402-3410.
29. Wang, B.; Li, Y.; Wu, N.; Lan, C. Q., CO₂ bio-mitigation using microalgae. *Applied Microbiology and Biotechnology* **2008**, 79 (5), 707-718.
30. Chisti, Y.; Yan, J. Y., Energy from algae: Current status and future trends Algal biofuels - A status report. *Appl. Energy* **2011**, 88 (10), 3277-3279.
31. Singh, N.; Dhar, D., Microalgae as second generation biofuel. A review. *Agronomy for Sustainable Development* **2011**, 31 (4), 605-629.
32. Sills, D. L.; Paramita, V.; Franke, M. J.; Johnson, M. C.; Akabas, T. M.; Greene, C. H.; Testert, J. W., Quantitative Uncertainty Analysis of Life Cycle Assessment for Algal Biofuel Production. *Environmental Science & Technology* **2013**, 47 (2), 687-694.
33. Liu, J.; Huang, H.; Chen, F., Microalgae as Feedstocks for Biodiesel Production. In *Biodiesel - Feedstocks and Processing Technologies*, InTech: 2011.
34. Solazyme Meeting the growing need for renewable fuels. <http://solazyme.com/fuels> (accessed 24th June 2014).
35. Ratledge, C.; Cohen, Z., Microbial and algal oils: Do they have a future for biodiesel or as commodity oils? *Lipid Technology* **2008**, 20 (7), 155-160.
36. Li, Q.; Du, W.; Liu, D., Perspectives of microbial oils for biodiesel production. *Applied Microbiology and Biotechnology* **2008**, 80 (5), 749-756.
37. Intriago, P., Marine Microorganisms: perspectives for getting involved in cellulosic ethanol. *AMB Express* **2012**, 2 (1), 46-46.
38. Ageitos, J.; Vallejo, J.; Veiga-Crespo, P.; Villa, T., Oily yeasts as oleaginous cell factories. *Applied Microbiology and Biotechnology* **2011**, 90 (4), 1219-1227.
39. Zhang, J.; Saerens, K. M. J.; Van Bogaert, I. N. A.; Soetaert, W., Vegetable oil enhances sophorolipid production by *Rhodotorula bogoriensis*. *Biotechnol. Lett.* **2011**, 33 (12), 2417-2423.
40. Carlos Mata-Gomez, L.; Cesar Montanez, J.; Mendez-Zavala, A.; Noe Aguilar, C., Biotechnological production of carotenoids by yeasts: an overview. *Microbial Cell Factories* **2014**, 13.
41. Johnson, E. A., Biotechnology of non-Saccharomyces yeasts-the basidiomycetes. *Applied Microbiology and Biotechnology* **2013**, 97 (17), 7563-7577.
42. Chuck, C. J.; Santomauro, F.; Sargeant, L. A.; Whiffin, F.; Chantasuban, T.; Ghaffar, N. R. A.; Wagner, J. L.; Scott, R. J., Liquid transport fuels from microbial yeasts – current and future perspectives. *Biofuels* **2014**, 5 (3), 293-311.
43. Johnson, E. A., Biotechnology of non-Saccharomyces yeasts-the ascomycetes. *Applied Microbiology and Biotechnology* **2013**, 97 (2), 503-517.
44. Eroshin, V. K.; Krylova, N. I., Efficiency of lipid-synthesis by yeasts. *Biotechnol. Bioeng.* **1983**, 25 (7), 1693-1700.
45. Kitcha, S.; Cheirsilp, B., Screening of Oleaginous Yeasts and Optimization for Lipid Production Using Crude Glycerol as a Carbon Source. In *9th Eco-Energy and Materials*

- Science and Engineering Symposium*, Yupapin, P. P.; PivsaArt, S.; Ohgaki, H., Eds. 2011; Vol. 9.
46. Pan, L.-X.; Yang, D.-F.; Li, S.; Wei, L.; Chen, G.-G.; Liang, Z.-Q., Isolation of the Oleaginous Yeasts from the Soil and Studies of Their Lipid-Producing Capacities. *Food Technol. Biotechnol.* **2009**, *47* (2), 215-220.
 47. Rattray, J. B. M., *Yeasts*. 1988; p 555-698.
 48. Amaretti, A.; Raimondi, S.; Sala, M.; Roncaglia, L.; De Lucia, M.; Leonardi, A.; Rossi, M., Single cell oils of the cold-adapted oleaginous yeast *Rhodotorula glacialis* DBVPG 4785. *Microbial Cell Factories* **2010**, *9*.
 49. Hansson, L.; Dostalek, M., Effect of culture conditions on fatty-acid composition in lipids produced by the yeast *Cryptococcus albidus* var. *albidus*. *Journal of the American Oil Chemists Society* **1986**, *63* (9), 1179-1184.
 50. Husain, S. S.; Hardin, M. M., Influence of carbohydrate and nitrogen sources upon lipid production by certain yeasts. *Food Research* **1952**, *17* (1), 60-66.
 51. Pedersen, T. A., Lipid formation in *Cryptococcus terricolus*. 1. Nitrogen nutrition and lipid formation. *Acta Chemica Scandinavica* **1961**, *15* (3), 651-&.
 52. Sitepu, I. R.; Ignatia, L.; Franz, A. K.; Wong, D. M.; Faulina, S. A.; Tsui, M.; Kanti, A.; Boundy-Mills, K., An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *Journal of Microbiological Methods* **2012**, *91* (2), 321-328.
 53. Kurtzman, C.; Fell, J. W.; Boekhout, T., *The Yeasts: A Taxonomic Study*. Elsevier Science: 2011.
 54. Yurkov, A. M.; Kemler, M.; Begerow, D., Assessment of yeast diversity in soils under different management regimes. *Fungal Ecology* **2012**, *5* (1), 24-35.
 55. Hainal, A. R.; Capraru, A. M.; Volf, I.; Popa, V. I., Lignin as a carbon source for the cultivation of some *Rhodotorula* species. *Cellulose Chemistry and Technology* **2012**, *46* (1-2), 87-96.
 56. Galafassi, S.; Cucchetti, D.; Pizza, F.; Franzosi, G.; Bianchi, D.; Compagno, C., Lipid production for second generation biodiesel by the oleaginous yeast *Rhodotorula graminis*. *Bioresource Technol* **2012**, *111*, 398-403.
 57. Zhang, G. C.; French, W. T.; Hernandez, R.; Alley, E.; Paraschivescu, M., Effects of furfural and acetic acid on growth and lipid production from glucose and xylose by *Rhodotorula glutinis*. *Biomass Bioenerg* **2011**, *35* (1), 734-740.
 58. Castoria, R.; Morena, V.; Caputo, L.; Panfili, G.; De Curtis, F.; De Cicco, V., Effect of the biocontrol yeast *Rhodotorula glutinis* strain LS11 on patulin accumulation in stored apples. *Phytopathology* **2005**, *95* (11), 1271-1278.
 59. Zhang, H.; Wang, L.; Ma, L.; Dong, Y.; Jiang, S.; Xu, B.; Zheng, X., Biocontrol of major postharvest pathogens on apple using *Rhodotorula glutinis* and its effects on postharvest quality parameters. *Biological Control* **2009**, *48* (1), 79-83.
 60. Patino-Vera, M.; Jimenez, B.; Balderas, K.; Ortiz, M.; Allende, R.; Carrillo, A.; Galindo, E., Pilot-scale production and liquid formulation of *Rhodotorula minuta*, a potential biocontrol agent of mango anthracnose. *Journal of Applied Microbiology* **2005**, *99* (3), 540-550.
 61. Bai, J.; Yao, H.; Fan, F.; Lin, M.; Zhang, L.; Ding, H.; Lei, F.; Wu, X.; Li, X.; Guo, J.; Qin, Z., Biosorption of uranium by chemically modified *Rhodotorula glutinis*. *Journal of Environmental Radioactivity* **2010**, *101* (11), 969-973.
 62. Bai, J.; Wu, X.; Fan, F.; Tian, W.; Yin, X.; Zhao, L.; Fan, F.; Li, Z.; Tian, L.; Qin, Z.; Guo, J., Biosorption of uranium by magnetically modified *Rhodotorula glutinis*. *Enzyme and Microbial Technology* **2012**, *51* (6-7), 382-387.
 63. Bai, J.; Li, Z.; Fan, F.; Wu, X.; Tian, W.; Yin, X.; Zhao, L.; Fan, F.; Tian, L.; Wang, Y.; Qin, Z.; Guo, J., Biosorption of uranium by immobilized cells of *Rhodotorula glutinis*. *Journal of Radioanalytical and Nuclear Chemistry* **2014**, *299* (3), 1517-1524.
 64. Katayamahirayama, K.; Tobita, S.; Hirayama, K., Degradation of phenol by yeast *Rhodotorula*. *Journal of General and Applied Microbiology* **1991**, *37* (2), 147-156.
 65. Krallish, I.; Gonta, S.; Savenkova, L.; Bergauer, P.; Margesin, R., Phenol degradation by immobilized cold-adapted yeast strains of *Cryptococcus terreus* and *Rhodotorula creatinivora*. *Extremophiles* **2006**, *10* (5), 441-449.

66. Cui, J. D.; Cui, L. L.; Zhang, S. P.; Zhang, Y. F.; Su, Z. G.; Ma, G. H., Hybrid Magnetic Cross-Linked Enzyme Aggregates of Phenylalanine Ammonia Lyase from *Rhodotorula glutinis*. *PLoS one* **2014**, *9* (5), e97221-e97221.
67. Zhu, L.; Cui, W.; Fang, Y.; Liu, Y.; Gao, X.; Zhou, Z., Cloning, expression and characterization of phenylalanine ammonia-lyase from *Rhodotorula glutinis*. *Biotechnol. Lett.* **2013**, *35* (5), 751-756.
68. El-Batal, A. I., Continuous production of L-phenylalanine by *Rhodotorula glutinis* immobilized cells using a column reactor. *Acta Microbiologica Polonica* **2002**, *51* (2), 153-169.
69. Frengova, G. I.; Beshkova, D. M., Carotenoids from *Rhodotorula* and *Phaffia*: yeasts of biotechnological importance. *Journal of Industrial Microbiology & Biotechnology* **2009**, *36* (2), 163-180.
70. Marova, I.; Carnecka, M.; Halienova, A.; Certik, M.; Dvorakova, T.; Haronikova, A., Use of several waste substrates for carotenoid-rich yeast biomass production. *Journal of Environmental Management* **2012**, *95*, S338-S342.
71. Moline, M.; Regina Flores, M.; Libkind, D.; del Carmen Dieguez, M.; Eugenia Farias, M.; van Broock, M., Photoprotection by carotenoid pigments in the yeast *Rhodotorula mucilaginosa*: the role of torularhodin. *Photochemical & Photobiological Sciences* **2010**, *9* (8), 1145-1151.
72. Saenge, C.; Cheirsilp, B.; Suksaroge, T. T.; Bourtoom, T., Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. *Process Biochemistry* **2011**, *46* (1), 210-218.
73. Yen, H.-W.; Yang, Y.-C.; Yu, Y.-H., Using crude glycerol and thin stillage for the production of microbial lipids through the cultivation of *Rhodotorula glutinis*. *Journal of bioscience and bioengineering* **2012**, *114* (4), 453-6.
74. Pan, J. G.; Kwak, M. Y.; Rhee, J. S., High-Density Cell-Culture of *Rhodotorula-Glutinis* Using Oxygen-Enriched Air. *Biotechnol. Lett.* **1986**, *8* (10), 715-718.
75. Sitepu, I. R.; Sestric, R.; Ignatia, L.; Levin, D.; German, J. B.; Gillies, L. A.; Almada, L. A. G.; Boundy-Mills, K. L., Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species. *Bioresource Technol* **2013**, *144*, 360-9.
76. Braunwald, T.; Schwemmlin, L.; Graeff-Honninger, S.; French, W. T.; Hernandez, R.; Holmes, W. E.; Claupein, W., Effect of different C/N ratios on carotenoid and lipid production by *Rhodotorula glutinis*. *Applied Microbiology and Biotechnology* **2013**, *97* (14), 6581-6588.
77. Latha, B. V. J., K.; Murali, H.S.; Manja, K.S., Influence of growth factors on carotenoid pigmentation of *Rhodotorula glutinis* DFR-PDY. *Indian Journal of Biotechnology* **2005**, *4*, 353-357.
78. Monsalve, L. N.; Cerrutti, P.; Galvagno, M. A.; Baldessari, A., *Rhodotorula minuta*-mediated bioreduction of 1,2-diketones. *Biocatalysis and Biotransformation* **2010**, *28* (2), 137-143.
79. Rustoy, E. M.; Cerrutti, P.; Galvagno, M. A.; Baldessari, A., An efficient biotransformation of dialkyl esters of 2-oxoglutaric acid by *Rhodotorula minuta* whole cells. *Biocatalysis and Biotransformation* **2008**, *26* (3), 204-209.
80. Fujii, T.; Ogawa, T.; Fukuda, H., Isobutene production by *Rhodotorula minuta*. *Applied Microbiology and Biotechnology* **1987**, *25* (5), 430-433.
81. Saxena, V.; Sharma, C. D.; Bhagat, S. D.; Saini, V. S.; Adhikari, D. K., Lipid and fatty acid biosynthesis by *Rhodotorula minuta*. *Journal of the American Oil Chemists Society* **1998**, *75* (4), 501-505.
82. Huang, C.; Chen, X.-f.; Xiong, L.; Chen, X.-d.; Ma, L.-l.; Chen, Y., Single cell oil production from low-cost substrates: The possibility and potential of its industrialization. *Biotechnology Advances* **2013**, *31* (2), 129-139.
83. Uçkun Kiran, E.; Trzcinski, A. P.; Ng, W. J.; Liu, Y., Bioconversion of food waste to energy: A review. *Fuel* **2014**, *134* (0), 389-399.
84. Chi, Z.; Zheng, Y.; Jiang, A.; Chen, S., Lipid Production by Culturing Oleaginous Yeast and Algae with Food Waste and Municipal Wastewater in an Integrated Process. *Applied Biochemistry and Biotechnology* **2011**, *165* (2), 442-453.

85. Ruan, Z.; Zanotti, M.; Zhong, Y.; Liao, W.; Ducey, C.; Liu, Y., Co-hydrolysis of lignocellulosic biomass for microbial lipid accumulation. *Biotechnol Bioeng* **2013**, *110* (4), 1039-49.
86. Papanikolaou, S.; Aggelis, G., Lipids of oleaginous yeasts. Part II: Technology and potential applications. *Eur. J. Lipid Sci. Technol.* **2011**, *113* (8), 1052-1073.
87. Wu, S.; Zhao, X.; Shen, H.; Wang, Q.; Zhao, Z. K., Microbial lipid production by *Rhodospiridium toruloides* under sulfate-limited conditions. *Bioresource Technol* **2011**, *102* (2), 1803-1807.
88. Paasche, E., Roles of nitrogen and phosphorus in coccolith formation in *Emiliania huxleyi* (Prymnesiophyceae). *European Journal of Phycology* **1998**, *33* (1), 33-42.
89. Evans, C. T.; Ratledge, C., The role of the mitochondrial NAD⁺ - isocitrate dehydrogenase in lipid-accumulation by the oleaginous yeast *Rhodospiridium toruloides* CBS-14. *Can J Microbiol* **1985**, *31* (9), 845-850.
90. Papanikolaou, S.; Aggelis, G., *Yarrowia lipolytica*: A model microorganism used for the production of tailor-made lipids. *Eur. J. Lipid Sci. Technol.* **2010**, *112* (6), 639-654.
91. Ratledge, C., Yeasts, moulds, algae and bacteria as sources of lipids. In *Technological Advances in Improved and Alternative Sources of Lipids*, Kamel, B. S.; Kakuda, Y., Eds. Springer US: 1994; pp 235-291.
92. Meng, X.; Yang, J.; Cao, Y.; Li, L.; Jiang, X.; Xu, X.; Liu, W.; Xian, M.; Zhang, Y., Increasing fatty acid production in *E. coli* by simulating the lipid accumulation of oleaginous microorganisms. *Journal of Industrial Microbiology and Biotechnology* **2011**, *38* (8), 919-925.
93. Ratledge, C.; Botham, P. A., Pathways of glucose metabolism in *Candida*-107, a lipid-accumulating yeast. *Journal of General Microbiology* **1977**, *102* (OCT), 391-395.
94. Bouvier-Nave, P.; Benveniste, P.; Oelkers, P.; Sturley, S. L.; Schaller, H., Expression in yeast and tobacco of plant cDNAs encoding acyl CoA : diacylglycerol acyltransferase. *European Journal of Biochemistry* **2000**, *267* (1), 85-96.
95. Cho, H.; Cronan, J. E., Jr., Defective export of a periplasmic enzyme disrupts regulation of fatty acid. *J Biol Chem* **1995**, *270* (9), 4216-4219.
96. Ratledge, C., Biochemistry, stoichiometry, substrates and economics. In *Single Cell Oil*, Moreton, R. S., Ed. Longman Scientific & Technical: London, 1988; pp 33-70.
97. Ratledge, C. E., C. T., Lipids and their metabolism. In *The yeast, 2nd edn.*, Academic, Ed. London, 1989; pp 367-455.
98. Uemura, H., Synthesis and production of unsaturated and polyunsaturated fatty acids in yeast: current state and perspectives. *Applied Microbiology and Biotechnology* **2012**, *95* (1), 1-12.
99. DeBell, R. M. a. J., R.C., Stereospecific analysis of major glycolipids of *Phycomyces blakesleeanus* sporangioophores and mycelium. *Journal of Bacteriology* **1975**, *124* (1), 220-224.
100. Halim, R.; Danquah, M. K.; Webley, P. A., Extraction of oil from microalgae for biodiesel production: A review. *Biotechnology Advances* **2012**, *30* (3), 709-732.
101. Change, D. o. E. a. C., Digest of UK Energy Statistics (DUKES). Chapter 1: Energy. 2012.
102. Government, H., The Carbon Plan: Delivering our low carbon future. 2011.
103. Atabani, A. E.; Silitonga, A. S.; Badruddin, I. A.; Mahlia, T. M. I.; Masjuki, H. H.; Mekhilef, S., A comprehensive review on biodiesel as an alternative energy resource and its characteristics. *Renew. Sust. Energ. Rev.* **2012**, *16* (4), 2070-2093.
104. Brennan, L.; Owende, P., Biofuels from microalgae - A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* **2010**, *14* (2), 557-577.
105. Union, C. o. t. E., Proposal for a Directive of the European Parliament and of the Council amending Directive 98/70/EC relating to the quality of petrol and diesel fuels and amending Directive 2009/28/EC on the promotion of the use of energy from renewable sources Brussels, 2014.
106. Koh, M. Y.; Mohd. Ghazi, T. I., A review of biodiesel production from *Jatropha curcas* L. oil. *Renewable and Sustainable Energy Reviews* **2011**, *15* (5), 2240-2251.

107. Canoira, L.; Alcantara, R.; Garcia-Martinez, J.; Carrasco, J., Biodiesel from Jojoba oil-wax: Transesterification with methanol and properties as a fuel. *Biomass Bioenerg* **2006**, *30* (1), 76-81.
108. Kulkarni, M. G.; Dalai, A. K., Waste cooking oil-an economical source for biodiesel: A review. *Ind Eng Chem Res* **2006**, *45* (9), 2901-2913.
109. *NNFCC Market Review: Biofuels*; 30 October 2013, 2013.
110. About our Biodiesel. <http://www.argentenergy.com/biodiesel/> (accessed 12 June 2014).
111. Taher, H., Al-Zuhair, S., Al-Marzouqi, A., Haik, Y., Farid, M., A Review of Enzymatic Transesterification of Microalgal Oil-Based Biodiesel Using Supercritical Technology. *Enzyme Research* **2011**, *2011*.
112. Schroder, O.; Bunger, J.; Munack, A.; Knothe, G.; Krahel, J., Exhaust emissions and mutagenic effects of diesel fuel, biodiesel and biodiesel blends. *Fuel* **2013**, *103*, 414-420.
113. Moreau, R. A.; Scott, K. M.; Haas, M. J., The identification and quantification of steryl glucosides in precipitates from commercial biodiesel. *Journal of the American Oil Chemists Society* **2008**, *85* (8), 761-770.
114. Van Hoed, V.; Zyaykina, N.; De Greyt, W.; Maes, J.; Verhe, R.; Demeestere, K., Identification and occurrence of steryl glucosides in palm and soy biodiesel. *Journal of the American Oil Chemists Society* **2008**, *85* (8), 701-709.
115. Knothe, G., A technical evaluation of biodiesel from vegetable oils vs. algae. Will algae-derived biodiesel perform? *Green Chemistry* **2011**, *13* (11), 3048-3065.
116. Knothe, G., Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Process Technol* **2005**, *86* (10), 1059-1070.
117. Knothe, G., "Designer" biodiesel: Optimizing fatty ester (composition) to improve fuel properties. *Energ Fuel* **2008**, *22* (2), 1358-1364.
118. Steen, E. J.; Kang, Y.; Bokinsky, G.; Hu, Z.; Schirmer, A.; McClure, A.; del Cardayre, S. B.; Keasling, J. D., Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* **2010**, *463* (7280), 559-562.
119. Schonborn, A.; Ladommatos, N.; Williams, J.; Allan, R.; Rogerson, J., The influence of molecular structure of fatty acid monoalkyl esters on diesel combustion. *Combustion and Flame* **2009**, *156* (7), 1396-1412.
120. Williams, P. J. I. B.; Laurens, L. M. L., Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics. *Energy & Environmental Science* **2010**, *3* (5), 554-590.
121. McCormick, R. L.; Graboski, M. S.; Alleman, T. L.; Herring, A. M., Impact of biodiesel source material and chemical structure on emissions of criteria pollutants from a heavy-duty engine. *Environmental Science & Technology* **2001**, *35* (9), 1742-1747.
122. Knothe, G.; Matheus, A. C.; Ryan, T. W., Cetane numbers of branched and straight-chain fatty esters determined in an ignition quality tester. *Fuel* **2003**, *82* (8), 971-975.
123. Hoekman, S. K.; Robbins, C., Review of the effects of biodiesel on NOx emissions. *Fuel Process Technol* **2012**, *96* (0), 237-249.
124. Tat, M. E.; Wang, P. S.; Van Gerpen, J. H.; Clemente, T. E., Exhaust emissions from an engine fueled with biodiesel from high-oleic soybeans. *Journal of the American Oil Chemists Society* **2007**, *84* (9), 865-869.
125. NREL, Impact of Biodiesel Impurities on the Performance and Durability of DOC, DPF and SCR Technologies. 2011.
126. Wahlen, B. D.; Morgan, M. R.; McCurdy, A. T.; Willis, R. M.; Morgan, M. D.; Dye, D. J.; Bugbee, B.; Wood, B. D.; Seefeldt, L. C., Biodiesel from Microalgae, Yeast, and Bacteria: Engine Performance and Exhaust Emissions. *Energ Fuel* **2013**, *27* (1), 220-228.
127. Liu, G.; Yan, B.; Chen, G., Technical review on jet fuel production. *Renewable and Sustainable Energy Reviews* **2013**, *25* (0), 59-70.
128. IPCC, Aviation and the global atmosphere. 1999.
129. Blakey, S.; Rye, L.; Wilson, C. W., Aviation gas turbine alternative fuels: A review. *Proceedings of the Combustion Institute* **2011**, *33*, 2863-2885.
130. Kallio, P.; Pásztor, A.; Akhtar, M. K.; Jones, P. R., Renewable jet fuel. *Current Opinion in Biotechnology* **2014**, *26* (0), 50-55.

131. Chevron Aviation Fuels Technical Review.
https://www.cgabusinessdesk.com/document/aviation_tech_review.pdf (accessed 23 June 2014).
132. Defence Standard 91-91: Turbine Fuel, Kerosene Type, Jet A-1. Ministry of Defence. : 2011.
133. Rye, L.; Blakey, S.; Wilson, C. W., Sustainability of supply or the planet: a review of potential drop-in alternative aviation fuels. *Energy & Environmental Science* **2010**, 3 (1), 17-27.
134. 2 million tons per year: A performing biofuels supply chain for EU aviation.
http://ec.europa.eu/energy/renewables/biofuels/doc/20130911_a_performing_biofuels_supply_chain.pdf (accessed 11 June 2014).
135. UK aviation forecasts 2011. Transport, D. f., Ed. HM Government: 2011.
136. European Advanced Biofuels Flight path Initiative.
http://ec.europa.eu/energy/renewables/biofuels/flight_path_en.htm (accessed 11 June 2014).
137. Verma, D.; Kumar, R.; Rana, B. S.; Sinha, A. K., Aviation fuel production from lipids by a single-step route using hierarchical mesoporous zeolites. *Energy & Environmental Science* **2011**, 4 (5), 1667-1671.
138. Kinder, J. D. *Evaluation of Bio-Derived Synthetic Paraffinic Kerosenes (Bio-SPKs)*; 2010.
139. Neste Neste Oil: Refining the future.
<http://www.nesteoil.com/default.asp?path=1,41,540,2384,18009> (accessed 20th June 2014).
140. Bailis, R. E.; Baka, J. E., Greenhouse Gas Emissions and Land Use Change from Jatropa Curcas-Based Jet Fuel in Brazil. *Environmental Science & Technology* **2010**, 44 (22), 8684-8691.
141. Wagner, S., EADS aircraft runs on algae biofuel. *Engineer* **2010**, JULY (Compendex).
142. AlgaeTec Algae. Tec and Lufthansa sign collaboration agreement to build biofuel production facility in Europe. <http://algaetec.com.au/news-room/press-releases/algae-tec-and-lufthansa-sign-collaboration-agreement-to-build-biofuel-production-facility-in-europe/> (accessed 19 September 2012).
143. ARA Why Readijet? <http://www.ara.com/fuels/Why-Readijet.html> (accessed 13th August 2014).
144. Solazyme Transformative technology. <http://solazyme.com/innovation/> (accessed 13th August 2014).
145. Sapphire The Sapphire Energy Story. <http://www.sapphireenergy.com/sapphire-renewable-energy> (accessed 13th August 2014).
146. Hill, K., Fats and oils as oleochemical raw materials. *Pure and Applied Chemistry* **2000**, 72 (7), 1255-1264.
147. Are we there yet? The positioning and repositioning of the algae industry.
<http://www.biofuelsdigest.com/bdigest/2014/06/29/are-we-there-yet-the-positioning-and-repositioning-of-the-algae-industry/> (accessed 29 June 2014).
148. SustainableBrands Solazyme and Unilever Sign Supply Agreement for Tailored Algal Oil.
http://www.sustainablebrands.com/news_and_views/collaboration/solazyme-unilever-sign-supply-agreement-tailored-algal-oil (accessed 24 May 2014).
149. Ecover Ecover and Algal Oil. <http://uk.ecover.com/en/why-ecover/algal-oil-the-alternative-to-palm-oil/> (accessed 24 May 2014).
150. Moreton, R. S., Modification of fatty acid composition of lipid accumulating yeasts with cyclopropene fatty acid desaturase inhibitors. *Applied Microbiology and Biotechnology* **1985**, 22 (1), 42-45.
151. Ykema, A.; Verbree, E.; Nijkamp, H. J.; Smit, H., Isolation and characterization of fatty acid auxotrophs from the oleaginous yeast *Apiotrichum curvatum*. *Applied Microbiology and Biotechnology* **1989**, 32 (1), 76-84.
152. Damude, H. G.; Zhang, H. X.; Farrall, L.; Ripp, K. G.; Tomb, J. F.; Hollerbach, D.; Yadav, N. S., Identification of bifunctional Delta 12/omega 3 fatty acid desaturases for improving the ratio of omega 3 to omega 6 fatty acids in microbes and plants. *P Natl Acad Sci USA* **2006**, 103 (25), 9446-9451.
153. Kuratko, C. N.; Salem, N., Jr., Docosahexaenoic acid from algal oil. *Eur. J. Lipid Sci. Technol.* **2013**, 115 (9), 965-976.

154. Velayutham, T.; Abd Majid, W.; Ng, B.; Gan, S., Effect of oleic acid content and chemical crosslinking on the properties of palm oil-based polyurethane coatings. *J Appl Polym Sci* **2013**, *129* (1), 415-421.
155. Montgomery, D. C., *Design and Analysis of Experiments*. 4th edition ed.; John Wiley: New York, 1997.
156. Bligh, E. G.; Dyer, W. J., A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **1959**, *37* (8), 911-917.
157. Doran, P. M., *Bioprocess Engineering Principles*. Elsevier Science: 1995.
158. *Wrap Household Food and Drink Waste in the United Kingdom 2012*; 2012.
159. Rosgaard, L.; Pedersen, S.; Langston, J.; Akerhielm, D.; Cherry, J. R.; Meyer, A. S., Evaluation of Minimal *Trichoderma reesei* Cellulase Mixtures on Differently Pretreated Barley Straw Substrates. *Biotechnol Progr* **2007**, *23* (6), 1270-1276.
160. Reese, E. T. L., H.S.; Downing, M., Quartermaster culture collection. *Farlowia* **1950**, *4*, 45-86.
161. Knothe, G.; Kenar, J. A., Determination of the fatty acid profile by H-1-NMR spectroscopy. *Eur. J. Lipid Sci. Technol.* **2004**, *106* (2), 88-96.
162. Wahlen, B. D.; Barney, B. M.; Seefeldt, L. C., Synthesis of Biodiesel from Mixed Feedstocks and Longer Chain Alcohols Using an Acid-Catalyzed Method. *Energ Fuel* **2008**, *22* (6), 4223-4228.
163. Mlickova, K.; Luo, Y.; d'Andrea, S.; Pec, P.; Chardot, T.; Nicaud, J. M., Acyl-CoA oxidase, a key step for lipid accumulation in the yeast *Yarrowia lipolytica*. *J Mol Catal B-Enzym* **2004**, *28* (2-3), 81-85.
164. Rattray, J. B. M.; Schibeci, A.; Kidby, D. K., Lipids of yeasts. *Bacteriological Reviews* **1975**, *39* (3), 197-231.
165. Ratledge, C., Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. *Biochimie* **2004**, *86* (11), 807-815.
166. Wu, S.; Hu, C.; Jin, G.; Zhao, X.; Zhao, Z. K., Phosphate-limitation mediated lipid production by *Rhodospiridium toruloides*. *Bioresource Technol* **2010**, *101* (15), 6124-6129.
167. Scrimgeour, C., Food Lipids - Chemistry, Nutrition, and Biotechnology, 2nd edn. by Casimir C. Akoh and David B. Min. *Eur. J. Lipid Sci. Technol.* **2003**, *105* (7), 381-381.
168. Demirbas, A., Fuel properties and calculation of higher heating values of vegetable oils. *Fuel* **1998**, *77* (9-10), 1117-1120.
169. Mandelli, D.; Jannini, M.; Buffon, R.; Schuchardt, U., Ethenolysis of esters of vegetable oils: Effect of B₂O₃ addition to Re₂O₇/SiO₂.Al₂O₃-SnBu₄ and CH₃ReO₃/SiO₂.Al₂O₃ metathesis catalysts. *Journal of the American Oil Chemists Society* **1996**, *73* (2), 229-232.
170. Donnelly, J., (Bio)-catalytic synthesis of a novel transport substitute from industrially produced fermentation products. University of Bath: 2014.
171. Hassan, M.; Blanc, P. J.; Granger, L. M.; Pareilleux, A.; Goma, G., Lipid production by an unsaturated fatty acid auxotroph of the oleaginous yeast *Apiotrichum curvatum* grown in single-stage continuous culture. *Applied Microbiology and Biotechnology* **1993**, *40* (4), 483-488.
172. Verwoert, I.; Ykema, A.; Valkenburg, J. A. C.; Verbree, E. C.; John, H.; Nijkamp, J.; Smit, H., Modification of the fatty acid composition in the lipids of the oleaginous yeast *Apiotrichum curvatum* by intraspecific spheroplast fusion. *Applied Microbiology and Biotechnology* **1989**, *32* (3), 327-333.
173. Davies, R. J.; Holdsworth, J. E.; Reader, S. L., The effect of low oxygen uptake rate on the fatty acid profile of the oleaginous yeast *Apiotrichum curvatum*. *Applied Microbiology and Biotechnology* **1990**, *33* (5), 569-573.
174. Suutari, M.; Liukkonen, K.; Laakso, S., Temperature adaptation in yeasts: The role of fatty acids. *Journal of General Microbiology* **1990**, *136*, 1469-1474.
175. Wu, S.; Hu, C.; Zhao, X.; Zhao, Z. K., Production of lipid from N-acetylglucosamine by *Cryptococcus curvatus*. *Eur. J. Lipid Sci. Technol.* **2010**, *112* (7), 727-733.
176. Bellou, S.; Moustogianni, A.; Makri, A.; Aggelis, G., Lipids Containing Polyunsaturated Fatty Acids Synthesized by Zygomycetes Grown on Glycerol. *Applied Biochemistry and Biotechnology* **2012**, *166* (1), 146-158.
177. Granger, L. M.; Perlot, P.; Goma, G.; Pareilleux, A., Kinetics of growth and fatty-acid production of *Rhodotorula glutinis*. *Applied Microbiology and Biotechnology* **1992**, *37* (1), 13-17.

178. Ferrante, G.; Kates, M., Pathways for desaturation of oleoyl chains in *Candida lipolytica*. *Canadian Journal of Biochemistry and Cell Biology* **1983**, *61* (11), 1191-1196.
179. Zhao, X.; Kong, X.; Hua, Y.; Feng, B.; Zhao, Z., Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi*. *Eur. J. Lipid Sci. Technol.* **2008**, *110* (5), 405-412.
180. Cho, D.; Chae, H.; Kim, E., Synthesis and characterization of a novel extracellular polysaccharide by *Rhodotorula glutinis*. *Applied Biochemistry and Biotechnology* **2001**, *95* (3), 183-193.
181. Yang, Z.; Li, L.; Hu, B.; Lin, L.; Wei, Y.; Ji, X.; Zhang, Q., Correlation between polyunsaturated fatty acids and cold adaptation of *Rhodotorula glutinis*. *Chinese Journal of Applied and Environmental Biology* **2014**, *20* (2), 233-237.
182. Ming, T. C.; Ramli, N.; Lye, O. T.; Said, M.; Kasim, Z., Strategies for decreasing the pour point and cloud point of palm oil products. *Eur. J. Lipid Sci. Technol.* **2005**, *107* (7-8), 505-512.
183. Tangsathitkulchai, C.; Sittichaitaweekul, Y.; Tangsathitkulchai, M., Temperature effect on the viscosities of palm oil and coconut oil blended with diesel oil. *Journal of the American Oil Chemists' Society* **2004**, *81* (4), 401-405.
184. Chuck, C. J.; Jenkins, R. W.; Bannister, C. D.; Han, L.; Lowe, J. P., Design and preliminary results of an NMR tube reactor to study the oxidative degradation of fatty acid methyl ester. *Biomass and Bioenergy* **2012**, *47* (0), 188-194.
185. Jenkins, R. W., Renewable liquid fuels from microbes and waste resources. University of Bath: 2014.
186. Hooijer, A.; Page, S.; Canadell, J. G.; Silvius, M.; Kwadijk, J.; Wosten, H.; Jauhiainen, J., Current and future CO₂ emissions from drained peatlands in Southeast Asia. *Biogeosciences* **2010**, *7* (5), 1505-1514.
187. OECD-FAO *Oilseeds and oilseed products*; 2013; pp 139-282.
188. Fargione, J.; Hill, J.; Tilman, D.; Polasky, S.; Hawthorne, P., Land clearing and the biofuel carbon debt. *Science* **2008**, *319* (5867), 1235-1238.
189. Zhao, X.; Hu, C.; Wu, S.; Shen, H.; Zhao, Z., Lipid production by *Rhodospiridium toruloides* Y4 using different substrate feeding strategies. *Journal of Industrial Microbiology & Biotechnology* **2011**, *38* (5), 627-632.
190. Hu, C.; Wu, S.; Wang, Q.; Jin, G.; Shen, H.; Zhao, Z. K., Simultaneous utilization of glucose and xylose for lipid production by *Trichosporon cutaneum*. *Biotechnol Biofuels* **2011**, *4*, 25.
191. Mason, T. J. Sonochemistry - beyond synthesis. <http://www.rsc.org/education/eic/issues/2009Sept/SonochemistryBeyondSynthesis.asp> (accessed 28th January).
192. Chisti, Y.; Mooyoung, M., Disruption of microbial cells for intracellular products. *Enzyme and Microbial Technology* **1986**, *8* (4), 194-204.
193. Chisti, Y., Sonobioreactors: using ultrasound for enhanced microbial productivity. *Trends in Biotechnology* **2003**, *21* (2), 89-93.
194. Hensirisak, P.; Parasukulsatid, P.; Agblevor, F. A.; Cundiff, J. S.; Velandar, W. H., Scale-up of microbubble dispersion generator for aerobic fermentation. *Appl Biochem Biotechnol* **2002**, *101* (3), 211-27.
195. Zhang, W.; Li, Z. J.; Agblevor, F. A., Microbubble fermentation of recombinant *Pichia pastoris* for human serum albumin production. *Process Biochemistry* **2005**, *40* (6), 2073-2078.
196. Weber, J.; Agblevor, F. A., Microbubble fermentation of *Trichoderma reesei* for cellulase production. *Process Biochemistry* **2005**, *40* (2), 669-676.
197. Nyborg, W. L., Ultrasonic microstreaming and related phenomena. *The British journal of cancer. Supplement* **1982**, *5*, 156-60.
198. Anderson, J. M., Effects of ultrasonic radiation on growth and fermentation in the yeast, *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **1953**, *11* (0), 122-137.
199. Matsuura, K.; Hirotsune, M.; Nunokawa, Y.; Satoh, M.; Honda, K., Acceleration of cell growth and ester formation by ultrasonic wave irradiation. *Journal of Fermentation and Bioengineering* **1994**, *77* (1), 36-40.
200. Sulaiman, A. Z.; Ajit, A.; Yunus, R. M.; Chisti, Y., Ultrasound-assisted fermentation enhances bioethanol productivity. *Biochemical Engineering Journal* **2011**, *54* (3), 141-150.

201. Chu, J.; Li, B. L.; Zhang, S. L.; Li, Y. R., On-line ultrasound stimulates the secretion and production of gentamicin by *Micromonospora echinospora*. *Process Biochemistry* **2000**, *35* (6), 569-572.
202. Schläfer, O.; Sievers, M.; Klotzbücher, H.; Onyeche, T. I., Improvement of biological activity by low energy ultrasound assisted bioreactors. *Ultrasonics* **2000**, *38* (1–8), 711-716.
203. Bati, N.; Hammond, E. G.; Glatz, B. A., Biomodification of fats and oils: Trials with *Candida lipolytica*. *J Am Oil Chem Soc* **1984**, *61* (11), 1743-1746.
204. Choi, S. Y.; Ryu, D. D. Y.; Rhee, J. S., Production of microbial lipid: Effects of growth rate and oxygen on lipid-synthesis and fatty acid composition of *Rhodotorula gracilis*. *Biotechnol. Bioeng.* **1982**, *24* (5), 1165-1172.
205. Yen, H.-W.; Zhang, Z., Effects of dissolved oxygen level on cell growth and total lipid accumulation in the cultivation of *Rhodotorula glutinis*. *Journal of bioscience and bioengineering* **2011**, *112* (1), 71-74.
206. Johnson, V.; Singh, M.; Saini, V. S.; Sista, V. R.; Yadav, N. K., Effect of pH on lipid accumulation by an oleaginous yeast: *Rhodotorula glutinis* IIP-30. *World J Microbiol Biotechnol* **1992**, *8* (4), 382-384.
207. Spotholz, C. H.; Litchfield, J. H.; Ordal, Z. J., The effect of pH, temperature and composition of the medium on growth characteristics of *Rhodotorula gracilis*. *Applied microbiology* **1956**, *4* (6), 285-288.
208. Symposium, B. M. S.; Boddy, L.; Marchant, R.; Read, D. J., *Nitrogen, Phosphorus, and Sulphur Utilization by Fungi: Symposium of the British Mycological Society Held at the University of Birmingham, April 1988*. Cambridge University Press: 1989.
209. Prusky, D.; McEvoy, J. L.; Leverentz, B.; Conway, W. S., Local Modulation of Host pH by *Colletotrichum* Species as a Mechanism to Increase Virulence. *Molecular Plant-Microbe Interactions* **2001**, *14* (9), 1105-1113.
210. Rose, A. H.; Harrison, J. S., *The Yeasts: Yeast Technology*. Elsevier Science: 1993.
211. Leelasart, B.; Bonaly, R., Effect of control of pH on the excretion of acid phosphatase by *Rhodotorula glutinis*. *Applied Microbiology and Biotechnology* **1988**, *29* (6), 579-585.
212. Finogenova, T. V.; Shishkanova, N. V.; Fausek, E. A.; Eremina, S. S., Biosynthesis of isocitric acid from ethanol by yeasts. *Applied Microbiology and Biotechnology* **1991**, *36* (2), 231-235.
213. Kotyk, A.; Lapathitis, G.; Křenková, Š., Glucose- and K⁺-induced acidification in different yeast species. *Folia Microbiologica* **1999**, *44* (3), 295-298.
214. Souza, M. A. A. T., M. J.; Brandao, R. L., New aspects of the glucose activation of the H⁺-ATPase in the yeast *Saccharomyces cerevisiae*. *Microbiology* **2001**, *147* (10), 2849-2855.
215. Martinez, C. e. a., Production of *Rhodotorula glutinis*: a yeast that secretes alpha-L-arabinofuranosidase. *Electron. J. Biotechnol.* **2006**, *9* (4).
216. Davies, R. J., Yeast oil from cheese whey-process development. . In *Single Cell Oil*, Moreton, R. S., Ed. Longman Scientific & Technical Press. : Harlow, UK., 1988; pp 99-146.
217. Ashokkumar, M.; Lee, J.; Kentish, S.; Grieser, F., Bubbles in an acoustic field: An overview. *Ultrasonics Sonochemistry* **2007**, *14* (4), 470-475.
218. Ratledge, C.; Hall, M. J., Oxygen demand by lipid accumulating yeasts in continuous culture. *Applied and Environmental Microbiology* **1977**, *34* (2), 230-231.
219. Jones, R. P.; Greenfield, P. F., Effect of carbon dioxide on yeast growth and fermentation. *Enzyme and Microbial Technology* **1982**, *4* (4), 210-222.
220. Cho, D. H.; Chae, H. J.; Kim, E. Y., Synthesis and characterization of a novel extracellular polysaccharide by *Rhodotorula glutinis*. *Applied Biochemistry and Biotechnology* **2001**, *95* (3), 183-193.
221. Sargeant, L. A.; Chuck, C. J.; Donnelly, J.; Bannister, C. D.; Scott, R. J., Optimising the lipid profile, to produce either a palm oil or biodiesel substitute, by manipulation of the culture conditions for *Rhodotorula glutinis*. *Biofuels* **2014**, *5* (1), 33-43.
222. Koutinas, A. A.; Vlysidis, A.; Pleissner, D.; Kopsahelis, N.; Lopez Garcia, I.; Kookos, I. K.; Papanikolaou, S.; Kwan, T. H.; Lin, C. S. K., Valorization of industrial waste and by-product streams via fermentation for the production of chemicals and biopolymers. *Chemical Society Reviews* **2014**, *43* (8), 2587-2627.
223. Lijó, L.; González-García, S.; Bacenetti, J.; Fiala, M.; Feijoo, G.; Lema, J. M.; Moreira, M. T., Life Cycle Assessment of electricity production in Italy from anaerobic co-digestion of pig slurry and energy crops. *Renewable Energy* **2014**, *68* (0), 625-635.

224. Matsakas, L.; Kekos, D.; Loizidou, M.; Christakopoulos, P., Utilization of household food waste for the production of ethanol at high dry material content. *Biotechnol Biofuels* **2014**, *7*.
225. Jorgensen, H.; Kristensen, J. B.; Felby, C., Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels Bioproducts & Biorefining-Biofpr* **2007**, *1* (2), 119-134.
226. Ragauskas, A. J.; Beckham, G. T.; Biddy, M. J.; Chandra, R.; Chen, F.; Davis, M. F.; Davison, B. H.; Dixon, R. A.; Gilna, P.; Keller, M.; Langan, P.; Naskar, A. K.; Saddler, J. N.; Tschaplinski, T. J.; Tuskan, G. A.; Wyman, C. E., Lignin Valorization: Improving Lignin Processing in the Biorefinery. *Science* **2014**, *344* (6185).
227. EC, Council Directive 1999/31/EC of 26 April 1999 on the landfill of waste **1999**.
228. Browne, J. D.; Murphy, J. D., Assessment of the resource associated with biomethane from food waste. *Appl. Energy* **2013**, *104*, 170-177.
229. IMechE *Global food: Waste not, want not*; London, 2013.
230. EC *Preparatory study on food waste across EU 27*; 2010.
231. Wrap Collection and recycling of food waste. <http://www.wrap.org.uk/content/collection-and-recycling-food-waste-0> (accessed 14th September 2014).
232. Yan, S.; Li, J.; Chen, X.; Wu, J.; Wang, P.; Ye, J.; Yao, J., Enzymatical hydrolysis of food waste and ethanol production from the hydrolysate. *Renewable Energy* **2011**, *36* (4), 1259-1265.
233. Lin, C. S. K.; Pfaltzgraff, L. A.; Herrero-Davila, L.; Mubofu, E. B.; Abderrahim, S.; Clark, J. H.; Koutinas, A. A.; Kopsahelis, N.; Stamatelatou, K.; Dickson, F.; Thankappan, S.; Mohamed, Z.; Brocklesby, R.; Luque, R., Food waste as a valuable resource for the production of chemicals, materials and fuels. Current situation and global perspective. *Energy & Environmental Science* **2013**, *6* (2), 426-464.
234. Hong, Y. S.; Yoon, H. H., Ethanol production from food residues. *Biomass and Bioenergy* **2011**, *35* (7), 3271-3275.
235. Wyman, C. E., What is (and is not) vital to advancing cellulosic ethanol. *Trends in Biotechnology* **2007**, *25* (4), 153-157.
236. Jurado, E.; Gavala, H. N.; Skiadas, I. V., Enhancement of methane yield from wheat straw, miscanthus and willow using aqueous ammonia soaking. *Environ Technol* **2013**, *34* (13-16), 2069-75.
237. Vavouraki, A. I.; Angelis, E. M.; Kornaros, M., Optimization of thermo-chemical hydrolysis of kitchen wastes. *Waste Management* **2013**, *33* (3), 740-745.
238. Vavouraki, A. I.; Volioti, V.; Kornaros, M. E., Optimization of thermo-chemical pretreatment and enzymatic hydrolysis of kitchen wastes. *Waste Management* **2014**, *34* (1), 167-173.
239. Pleissner, D.; Kwan, T. H.; Lin, C. S. K., Fungal hydrolysis in submerged fermentation for food waste treatment and fermentation feedstock preparation. *Bioresource Technol* **2014**, *158*, 48-54.
240. Kádár, Z.; Szengyel, Z.; Réczey, K., Simultaneous saccharification and fermentation (SSF) of industrial wastes for the production of ethanol. *Industrial Crops and Products* **2004**, *20* (1), 103-110.
241. Öhgren, K.; Bura, R.; Lesnicki, G.; Saddler, J.; Zacchi, G., A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochemistry* **2007**, *42* (5), 834-839.
242. Palmqvist, E.; Hahn-Hägerdal, B., Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource Technol* **2000**, *74* (1), 25-33.
243. Olson, D. G.; McBride, J. E.; Joe Shaw, A.; Lynd, L. R., Recent progress in consolidated bioprocessing. *Current Opinion in Biotechnology* **2012**, *23* (3), 396-405.
244. Santamauro, F.; Whiffin, F.; Scott, R.; Chuck, C., Low-cost lipid production by an oleaginous yeast cultured in non-sterile conditions using model waste resources. *Biotechnol Biofuels* **2014**, *7* (1), 34.
245. Bettiga, M.; Hahn-Hägerdal, B.; Gorwa-Grauslund, M. F., Comparing the xylose reductase/xylitol dehydrogenase and xylose isomerase pathways in arabinose and xylose fermenting *Saccharomyces cerevisiae* strains. *Biotechnol Biofuels* **2008**, *1*.
246. Cate, J. H. D.; Ha, S. J.; Galazka, J. M.; Kim, S. R.; Choi, J. H.; Yang, X. M.; Seo, J. H.; Glass, N. L.; Jin, Y. S., Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *P Natl Acad Sci USA* **2011**, *108* (2), 504-509.

247. Jeffries, T. W.; Jin, Y. S., Ethanol and thermotolerance in the bioconversion of xylose by yeasts. *Advances in Applied Microbiology*, Vol 47 **2000**, 47, 221-268.
248. Aduseopoku, J.; Mitchell, W. J., Diauxic growth of *Clostridium thermosaccharolyticum* on glucose and xylose. *Fems Microbiol Lett* **1988**, 50 (1), 45-49.
249. Janda, S.; Kotyk, A., Some features of carbohydrate metabolism in *Rhodotorula glutinis*. *Folia Microbiologica* **1972**, 17 (6), 461-8.
250. Chen, X.; Li, Z.; Zhang, X.; Hu, F.; Ryu, D. Y.; Bao, J., Screening of Oleaginous Yeast Strains Tolerant to Lignocellulose Degradation Compounds. *Applied Biochemistry and Biotechnology* **2009**, 159 (3), 591-604.
251. Huang, C.; Chen, X.-f.; Xiong, L.; Yang, X.-y.; Chen, X.-d.; Ma, L.-l.; Chen, Y., Microbial oil production from corn cob acid hydrolysate by oleaginous yeast *Trichosporon coremiiforme*. *Biomass and Bioenergy* **2013**, 49 (0), 273-278.
252. Sitepu, I.; Selby, T.; Lin, T.; Zhu, S.; Boundy-Mills, K., Carbon source utilization and inhibitor tolerance of 45 oleaginous yeast species. *J Ind Microbiol Biotechnol* **2014**, 41 (7), 1061-70.
253. Dai, C. C.; Tao, J.; Xie, F.; Dai, Y. J.; Zhao, M., Biodiesel generation from oleaginous yeast *Rhodotorula glutinis* with xylose assimilating capacity. *Afr. J. Biotechnol.* **2007**, 6 (18), 2130-2134.
254. Lucas, C.; Vanuden, N., Transport of hemicellulose monomers in the xylose-fermenting yeast *Candida shehatae*. *Applied Microbiology and Biotechnology* **1986**, 23 (6), 491-495.
255. Leandro, M. J.; Goncalves, P.; Spencer-Martins, I., Two glucose/xylose transporter genes from the yeast *Candida intermedia*: first molecular characterization of a yeast xylose-H⁺ symporter. *Biochem J* **2006**, 395 (3), 543-9.
256. Wiebe, M. G.; Koivuranta, K.; Penttilä, M.; Ruohonen, L., Lipid production in batch and fed-batch cultures of *Rhodospiridium toruloides* from 5 and 6 carbon carbohydrates. *Bmc Biotechnology* **2012**, 12.
257. Yu, X.; Zheng, Y.; Dorgan, K. M.; Chen, S., Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. *Bioresource Technol* **2011**, 102 (10), 6134-6140.
258. Palmqvist, E.; Grage, H.; Meinander, N. Q.; Hahn-Hagerdal, B., Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. *Biotechnol. Bioeng.* **1999**, 63 (1), 46-55.
259. Viikari, L.; Vehmaanpera, J.; Koivula, A., Lignocellulosic ethanol: From science to industry. *Biomass Bioenergy* **2012**, 46, 13-24.
260. Saxena, R. C.; Adhikari, D. K.; Goyal, H. B., Biomass-based energy fuel through biochemical routes: A review. *Renewable and Sustainable Energy Reviews* **2009**, 13 (1), 167-178.
261. Chandel, A. K.; da Silva, S. S.; Singh, O. V., Detoxification of Lignocellulose Hydrolysates: Biochemical and Metabolic Engineering Toward White Biotechnology. *Bioenergy Res* **2013**, 6 (1), 388-401.
262. Larsson, S.; Palmqvist, E.; Hahn-Hägerdal, B.; Tengborg, C.; Stenberg, K.; Zacchi, G.; Nilvebrant, N.-O., The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme and Microbial Technology* **1999**, 24 (3-4), 151-159.
263. Brosse, N.; Dufour, A.; Meng, X. Z.; Sun, Q. N.; Ragauskas, A., Miscanthus: a fast-growing crop for biofuels and chemicals production. *Biofuels Bioproducts & Biorefining-Biofpr* **2012**, 6 (5), 580-598.
264. Williams, P. R.; Inman, D.; Aden, A.; Heath, G. A., Environmental and sustainability factors associated with next-generation biofuels in the U.S.: what do we really know? *Environ Sci Technol* **2009**, 43 (13), 4763-75.
265. Murnen, H. K.; Balan, V.; Chundawat, S. P.; Bals, B.; Sousa Lda, C.; Dale, B. E., Optimization of ammonia fiber expansion (AFEX) pretreatment and enzymatic hydrolysis of *Miscanthus x giganteus* to fermentable sugars. *Biotechnol Prog* **2007**, 23 (4), 846-50.
266. Sengar, G.; Sharma, H. K., Food caramels: a review. *Journal of food science and technology* **2014**, 51 (9), 1686-96.
267. Carballa, M.; Duran, C.; Hospido, A., Should We Pretreat Solid Waste Prior to Anaerobic Digestion? An Assessment of Its Environmental Cost. *Environmental Science & Technology* **2011**, 45 (24), 10306-10314.
268. Jönsson, L.; Alriksson, B.; Nilvebrant, N.-O., Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol Biofuels* **2013**, 6 (1), 1-10.

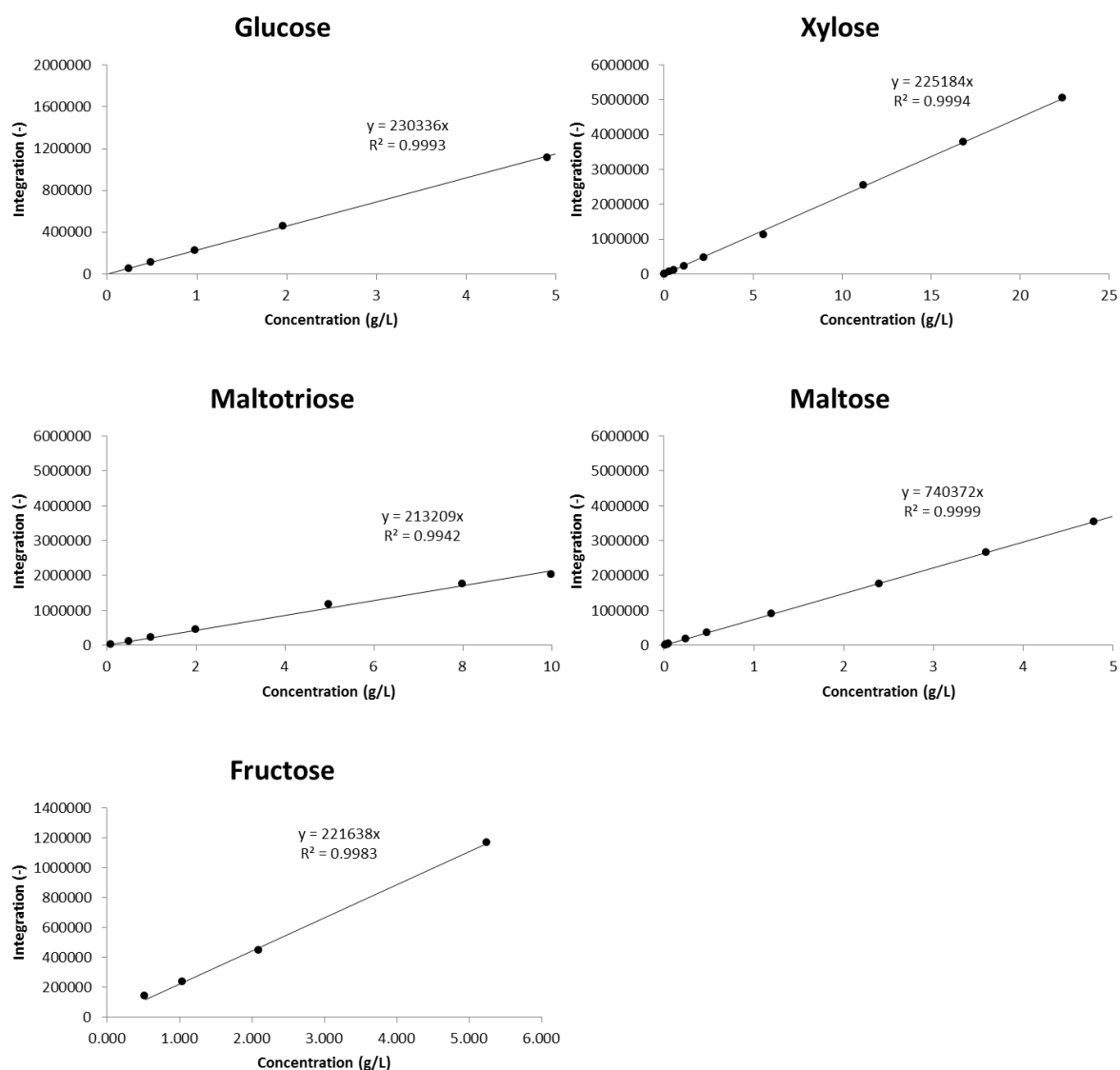
269. Alriksson, B.; Horvath, I. S.; Sjode, A.; Nilvebrant, N. O.; Jonsson, L. J., Ammonium hydroxide detoxification of spruce acid hydrolysates. *Appl Biochem Biotechnol* **2005**, *124*, 911-22.
270. Van Zyl, C.; Prior, B. A.; Du Preez, J. C., Production of ethanol from sugar cane bagasse hemicellulose hydrolyzate by *Pichia stipitis*. *Applied Biochemistry and Biotechnology* **1988**, *17* (1-3), 357-369.
271. Wang, C.; Chen, L.; Rakesh, B.; Qin, Y.; Lv, R., Technologies for extracting lipids from oleaginous microorganisms for biodiesel production. *Front. Energy* **2012**, *6* (3), 266-274.
272. Guedes, A. C.; Amaro, H. M.; Malcata, F. X., Microalgae As Sources of High Added-Value Compounds-A Brief Review of Recent Work. *Biotechnol Progr* **2011**, *27* (3), 597-613.
273. Lee, S.; Yoon, B.-D.; Oh, H.-M., Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. *Biotechnology Techniques* **1998**, *12* (7), 553-556.
274. Balasubramanian, S.; Allen, J. D.; Kanitkar, A.; Boldor, D., Oil extraction from *Scenedesmus obliquus* using a continuous microwave system - design, optimization, and quality characterization. *Bioresource Technol* **2011**, *102* (3), 3396-3403.
275. Achten, W. M. J.; Verchot, L.; Franken, Y. J.; Mathijs, E.; Singh, V. P.; Aerts, R.; Muys, B., *Jatropha* bio-diesel production and use. *Biomass and Bioenergy* **2008**, *32* (12), 1063-1084.
276. Lewis, T.; Nichols, P. D.; McMeekin, T. A., Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. *Journal of Microbiological Methods* **2000**, *43* (2), 107-116.
277. Lee, J.-Y.; Yoo, C.; Jun, S.-Y.; Ahn, C.-Y.; Oh, H.-M., Comparison of several methods for effective lipid extraction from microalgae. *Bioresource Technol* **2010**, *101*, S75-S77.
278. Hejazi, M. A.; Wijffels, R. H., Milking of microalgae. *Trends in Biotechnology* **2004**, *22* (4), 189-194.
279. Khoomrung, S.; Chumnpanpuen, P.; Jansa-ard, S.; Nookaew, I.; Nielsen, J., Fast and accurate preparation fatty acid methyl esters by microwave-assisted derivatization in the yeast *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* **2012**, *94* (6), 1637-1646.
280. Kaufmann, B.; Christen, P., Recent extraction techniques for natural products: Microwave-assisted extraction and pressurised solvent extraction. *Phytochemical Analysis* **2002**, *13* (2), 105-113.
281. Patil, P. D.; Gude, V. G.; Mannarswamy, A.; Cooke, P.; Munson-McGee, S.; Nirmalakhandan, N.; Lammers, P.; Deng, S., Optimization of microwave-assisted transesterification of dry algal biomass using response surface methodology. *Bioresource Technol* **2011**, *102* (2), 1399-1405.
282. Koutinas, A. A.; Chatzifragkou, A.; Kopsahelis, N.; Papanikolaou, S.; Kookos, I. K., Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. *Fuel* **2014**, *116* (0), 566-577.
283. Vicente, G.; Martinez, M.; Aracil, J., Integrated biodiesel production: a comparison of different homogeneous catalysts systems. *Bioresource Technol* **2004**, *92* (3), 297-305.
284. Freedman, B.; Pryde, E. H.; Mounts, T. L., Variables affecting the yields of fatty acid esters from transesterified vegetable oils. *Journal of the American Oil Chemists Society* **1984**, *61* (10), 1638-1643.
285. Goff, M.; Bauer, N.; Lopes, S.; Sutterlin, W.; Suppes, G., Acid-catalyzed alcoholysis of soybean oil. *Journal of the American Oil Chemists' Society* **2004**, *81* (4), 415-420.
286. Carrasco-Pancorbo, A.; Navas-Iglesias, N.; Cuadros-Rodríguez, L., From lipid analysis towards lipidomics, a new challenge for the analytical chemistry of the 21st century. Part I: Modern lipid analysis. *TrAC Trends in Analytical Chemistry* **2009**, *28* (3), 263-278.
287. Perin, G.; Alvaro, G.; Westphal, E.; Viana, L. H.; Jacob, R. G.; Lenardao, E. J.; D'Oca, M. G. M., Transesterification of castor oil assisted by microwave irradiation. *Fuel* **2008**, *87* (12), 2838-2841.
288. Liu, B.; Zhao, Z., Biodiesel production by direct methanolysis of oleaginous microbial biomass. *J Chem Technol Biot* **2007**, *82* (8), 775-780.
289. Cravotto, G.; Boffa, L.; Mantegna, S.; Perego, P.; Avogadro, M.; Cintas, P., Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. *Ultrasonics Sonochemistry* **2008**, *15* (5), 898-902.
290. Chen, K.-S.; Lin, Y.-C.; Hsu, K.-H.; Wang, H.-K., Improving biodiesel yields from waste cooking oil by using sodium methoxide and a microwave heating system. *Energy* **2012**, *38* (1), 151-156.

291. Azcan, N.; Danisman, A., Microwave assisted transesterification of rapeseed oil. *Fuel* **2008**, 87 (10-11), 1781-1788.
292. Frankel, E. N., Lipid oxidation - Mechanisms, products and biological significance. *Journal of the American Oil Chemists Society* **1984**, 61 (12), 1908-1917.
293. Abdulkadir, S.; Tsuchiya, M., One-step method for quantitative and qualitative analysis of fatty acids in marine animal samples. *Journal of Experimental Marine Biology and Ecology* **2008**, 354 (1), 1-8.
294. Nurfitri, I.; Maniam, G. P.; Hindryawati, N.; Yusoff, M. M.; Ganesan, S., Potential of feedstock and catalysts from waste in biodiesel preparation: A review. *Energy Conversion and Management* **2013**, 74, 395-402.
295. Davies, R. J., Scale Up of Yeast Oil Technology. In *Industrial Applications of Single Cell Oils*, AOCS Publishing: 1992.
296. Neste Oil inaugurates Europe's first pilot plant for producing microbial oil from waste and residues. <http://www.nesteoil.com/default.asp?path=1,41,540,1259,1260,18523,20202> (accessed 27th August 2014).
297. EC, Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain Directives. 2008.
298. Recyclenow Food waste: How it is recycled. <http://www.recyclenow.com/facts-figures/how-it-recycled/food-waste> (accessed 14th September 2014).
299. Zhang, R.; El-Mashad, H. M.; Hartman, K.; Wang, F.; Liu, G.; Choate, C.; Gamble, P., Characterization of food waste as feedstock for anaerobic digestion. *Bioresource Technol* **2007**, 98 (4), 929-935.
300. Evangelisti, S.; Lettieri, P.; Borello, D.; Clift, R., Life cycle assessment of energy from waste via anaerobic digestion: A UK case study. *Waste Management* **2014**, 34 (1), 226-237.
301. Zhang, C.; Su, H.; Baeyens, J.; Tan, T., Reviewing the anaerobic digestion of food waste for biogas production. *Renewable and Sustainable Energy Reviews* **2014**, 38 (0), 383-392.
302. Kim, J. H.; Lee Jc Fau - Pak, D.; Pak, D., Feasibility of producing ethanol from food waste. *Waste Management* **2011**, 31, 2121-2125.
303. Pleissner, D.; Lam, W. C.; Sun, Z.; Lin, C. S. K., Food waste as nutrient source in heterotrophic microalgae cultivation. *Bioresource Technol* **2013**, 137 (0), 139-146.
304. Green, D. W., *Perry's chemical engineers' handbook*. McGraw-hill New York: 2008; Vol. 796.
305. Bricout, S. *HEFA from Rhodotorula glutinis and Miscanthus giganteus: energy and GHG balance assessment*; Suresnes, 2012.
306. Nonus, M., Cognart, P., Kergoat, F., Lebeault, J.-M., Fermenteurs industriels : Conception et réalisation. *Techniques de l'ingénieur*. 2008.
307. Bakx, T., Membrez, Y., Mottet, A., Joss, A., Boehler, M., Etat de l'art des méthodes (rentables) pour l'élimination, la concentration ou la transformation de l'azote pour les installations de biogaz agricoles de taille petite/moyenne. *énergétique*, S. r., Ed. l'Office Fédéral de L'Energie: 2009.
308. Diagouraga, F., Analyse de cycle de vie - Biocarburants - Levures Oléagineuses. Suresnes, 2011.
309. Lardon, L.; Hélias, A.; Sialve, B.; Steyer, J.-P.; Bernard, O., Life-Cycle Assessment of Biodiesel Production from Microalgae. *Environmental Science & Technology* **2009**, 43 (17), 6475-6481.
310. Curry, N.; Pillay, P., Biogas prediction and design of a food waste to energy system for the urban environment. *Renewable Energy* **2012**, 41, 200-209.
311. Borjesson, P.; Berglund, M., Environmental systems analysis of biogas systems - Part 1: Fuel-cycle emissions. *Biomass Bioenergy* **2006**, 30 (5), 469-485.
312. Airbus, Industrial Ecology of Earth Resources: Management of solid wastes.
313. *EIA Annual Energy Outlook 2014 with projections to 2040*; 2014.
314. Silva, M. R. Q.; Naik, T. R., *Review of composting and anaerobic digestion of municipal solid waste and a methodological proposal for a mid-size city*. 2007; p 631-643.
315. Post, M. J., Cultured beef: medical technology to produce food. *Journal of the Science of Food and Agriculture* **2014**, 94 (6), 1039-1041.
316. BeyondMeat Beyond Meat. <http://beyondmeat.com/> (accessed 23rd September 2014).

317. ExoProtein Exo made with cricket flour: The future of protein. <http://exoprotein.com/pages/why-crickets> (accessed 23rd September 2014).
318. (a) AquaBounty Biotechnology to improve aquaculture productivity. <http://aquabounty.com/>; (b) Espinosa-Gonzalez, I.; Parashar, A.; Bressler, D. C., Heterotrophic growth and lipid accumulation of *Chlorella protothecoides* in whey permeate, a dairy by-product stream, for biofuel production. *Bioresource Technol* **2014**, *155*, 170-176.

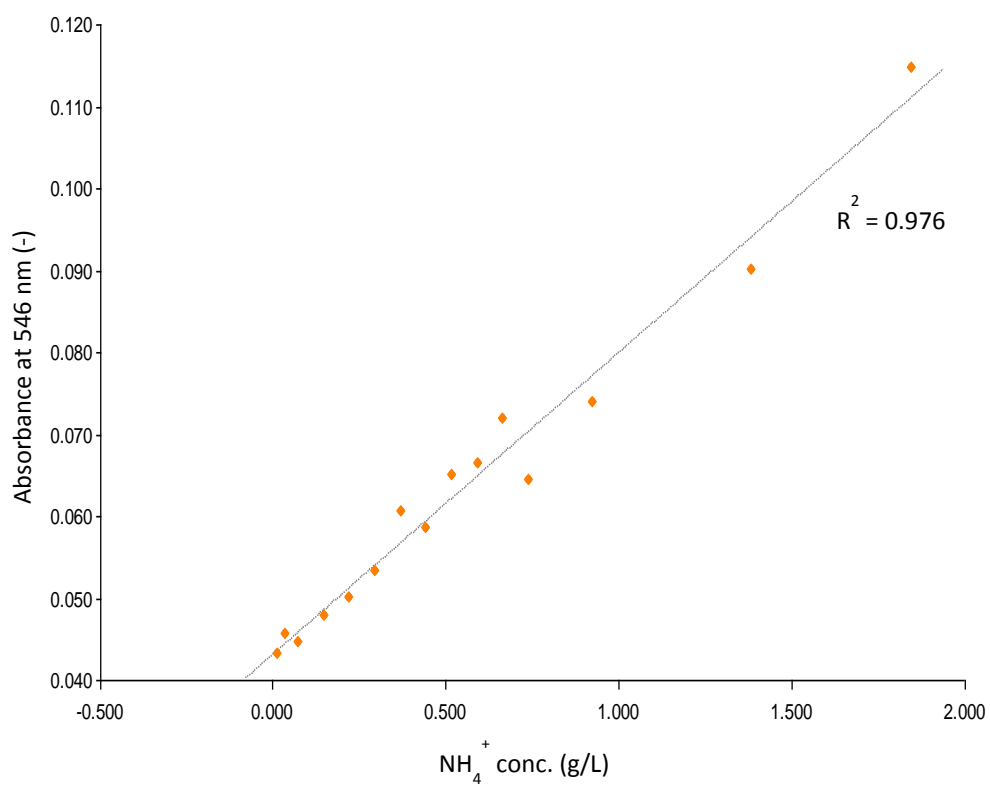
Appendix A

Calibration curves for the sugars analysed by HPLC.



Appendix B

Calibration curve for the colourimetric determination of ammonium in solution
(Radox Laboratories, Crumlin, UK).



Appendix C

Published journal articles arising and associated to the research undertaken in this thesis (copies of the manuscripts are provided at the end of this Appendix).

L. A. Sargeant, C. J. Chuck, J. Donnelly, C. D. Bannister, R. J. Scott, **2014**, Optimizing the lipid profile, to produce either a palm oil or biodiesel substitute, by manipulation of the culture conditions for *Rhodotorula glutinis*. *Biofuels*, 5 **(1)**, 33-34.

C. J. Chuck, D. Lou-Hing, R. Dean, L. A. Sargeant, R. J. Scott, R. W. Jenkins. **2014**. Simultaneous microwave extraction and synthesis of fatty acid methyl ester from the oleaginous yeast *Rhodotorula glutinis*. *Energy*. 69, 446-454.

C. J. Chuck, F. Santomauro, L. A. Sargeant, F. Whiffin, T. Chantasuban, N. R. A. Ghaffer, J. L. Wagner, R. J. Scott. **2014**, Liquid transport fuels from microbial yeasts – current and future perspectives. *Biofuels*. 5 **(3)** 293-311.

M. Harkins, D. Lou-Hing, L. A. Sargeant, C. J. Chuck, **2013**, Effect of bacterial invasion on the growth and lipid production of the oleaginous yeast *Rhodotorula glutinis*. *Journal of Technology Innovations in Renewable Energy*, 2, **(3)**, 220-230.